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Isolation of *Pseudomonas aeruginosa* phages and their application for the analysis of lipopolysaccharides

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Gonna stand my ground
And I won't back down

Tom Petty

Table of Contents

Abbreviations

ALA	5-aminolevulinic acid
APS	ammonium peroxidisulfate
BLAST	basic local alignment search tool
bp	base pair
C	Celsius (℃)
CF	Cystic fibrosis
CFU	colony forming unit
ddNTP	didesoxy nucleotide triphosphate
dH_2O	deionized water
DMSO	dimethylsulfoxid
DNA	desoxyribonucleic acid
dNTP	desoxy nucleotide triphosphate
ds	double stranded
EDTA	ethylenediamine tetraacetic acid
et al.	*et alteri* (and others)
e. g.	*exempli gratia* (for example)
FACS	fluorescence activated cell sorting
Fig.	figure
fwd	forward
g	centrifugation: earth gravity (x g); weight: gram
h	hour
k	kilo
L	liter
λ	wavelength
LB	Luria Bertani
LPS	lipopolysaccharide
m	milli
M	molar (mol/L)
μ	micro

MIC	minimal inhibitory concentration
min	minute
MOI	multiplicity of infection
nm	nanometer
rpm	rotation per minute
OD $_{\lambda}$	optical density at a wavelength λ in nm
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethyleneglycol
PFU	plaque forming unit
rev	reverse
ORF	open reading frame
ROS	reactive oyxgen species
RT	room temperature
s	second
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tab.	table
TEMED	tetramethylen diamine
tris	Tris-(hydroxymethyl)-aminomethane
U	unit
V	volt
v/v	volume per volume
WT	wild type
w/v	weight per volume

Zusammenfassung

Zwei lytische *Pseudomonas aeruginosa* spezifische Phagen mit weitem Wirtsspektrum, JG004 und JG024, wurden isoliert und charakterisiert. Beide Phagen gehören zur Familie *Myoviridae* und erkennen das Lipopolysaccharid des Wirtes als Rezeptor. Die Dauer des Infektionszyklusses und das Wirtsspektrum wurden untersucht. JG004 benötigt ungefähr 15 min um einen Infektionszyklus zu durchlaufen, Phage JG024 benötigt ungefähr 25 min. Weiterhin können beide Phagen ein breites Spektrum unterschiedlicher *P. aeruginosa* Umwelt- und klinischer Isolate, sowie Stämme mit einem mukoiden Phänotyp lysieren. Die Charakterisierung wurde mit der Sequenzierung beider Phagengenome vervollständigt. Phage JG004 zeigte nur geringe Ähnlichkeiten zu bisher beschriebenen Phagen. Es konnte gezeigt werden, dass der Phage von der Transkriptions- sowie DNA Replikationsmaschinerie des Wirtes unabhängig ist, aber auf die Spermidin Synthese des Wirtes angewiesen ist. Das Genom von JG004 weist zwölf tRNAs auf, die offensichtlich für eine optimierte Translation der Phagen mRNA benötigt werden. Phage JG024 wurde über den Genomvergleich als ein neues Mitglied der PB1-ähnlichen Phagen identifiziert.

LPS-spezifische Phagen ermöglichen die Untersuchung verschiedener Fragestellungen zum bakteriellen LPS. In diesem Teil der Arbeit wurde die Rolle des verkürzten LPS bei multiresistenten *P. aeruginosa* Stämmen untersucht. Ein Phagen-basiertes *in vitro* System wurde etabliert, um *P. aeruginosa* Stämme mit verändertem LPS zu identifizieren. Es wurde bewiesen, dass das verkürzte LPS aufgrund des Antibiotikastresses ensteht und zur Antibiotikatoleranz beiträgt, indem es die Toleranz gegenüber oxidativem Stress erhöht. Mutationen in dem Gen PA5001, welches für eine mögliche Glykosyltransferase codiert, konnten als Ursache für das verkürzte LPS identifiziert werden. Charakterisierung einer *P. aeruginosa* PA5001 Deletionsmutante bestätigte, dass die Verkürzung des LPS die Toleranz gegenüber oxidativem Stress erhöht. Eine mögliche Beteiligung des Lipoproteins OprI an dem Mechanismus der Stress Toleranz wurde diskutiert.

Diese Arbeit trägt dazu bei, die Biologie von *P. aeruginosa* spezifischen Phagen, sowie Antiobiotika induzierte LPS Veränderungen in *P. aeruginosa* zu verstehen.

Summary

Two new lytic *Pseudomonas aeruginosa* specific broad host range phages, JG004 and JG024, were isolated and characterized. Both phages belong to the family *Myoviridae* and recognize the lipopolysaccharide layer as host receptor. The duration of the infection cycle and the host range were determined. JG004 needs approximately 15 min for one complete infection cycle, phage JG024 needs approximately 25 min. Both phages infect a broad variety of different *P. aeruginosa* strains, isolated from the environment or clinic. Even strains with mucoid phenotype were lysed by these phages. The characterization was completed by DNA sequence determination of both phage genomes. Phage JG004 showed low similarities to other already sequenced phages. This phage is independent from the host transcriptional and DNA replication machinery but depends on spermidine synthesis of the host. The JG004 genome encodes twelve tRNAs, most likely for the improvement of phage mRNA translation. Phage JG024 was identified as a new member of the described PB1-like phages.

LPS-specific phages are suitable tools for the investigation of various questions regarding bacterial LPS. In this study, we investigated the impact of truncated LPS on antibiotic tolerance of multiresistant *P. aeruginosa* strains. A phage based *in vitro* system was established to isolate and investigate *P. aeruginosa* strains with variation in the LPS structure. We demonstrated that truncated LPS emerges as a consequence of antibiotic stress. Modified LPS contributes to the antibiotic tolerance by enhacing the tolerance to oxidative stress. Moreover, we identified mutations in the gene PA5001, a probable glycosyltransferase, as the cause for the LPS alterations. A *P. aeruginosa* PA5001 deletion mutant was used to confirm our results that truncation of the LPS leads to oxidative stress tolerance. The possible involvement of the lipoprotein OprI in the mechanism of the stress tolerance was discussed. In summary, this thesis contributes to the understanding of the biology of *P. aeruginosa* specific phages and to antibiotic induced LPS alteration of *P. aeruginosa*.

1 Introduction

1.1 General Phage Biology

Bacteriophages or phages in general are viruses that infect specifically bacteria. Like other viruses they are obligate parasites using a host organism to multiply and spread. A phage particle consists of a nucleic acid, DNA or RNA, which is enclosed in a protein or lipoprotein coat. The envelope itself protects the genetic information and is involved in the adsorption to the host (Guttman *et al.*, 2005).

There are 17 officially accepted and 3 described families of prokaryotic viruses (Ackermann, 2007) as shown in Table 1. Since there is no numerical taxonomy for viruses the classification for phages is based on the morphology and the nature of its nucleic acid (Clokie & Kropinski, 2009). Phages can have RNA or DNA, both single stranded (ss) or double stranded (ds) whereas only two phage families exhibit RNA as genetic material (Tab. 1).

The infection process starts with the adsorption of the phage to the host. Thereby, the phage binds reversible to a specific surface structure on their target bacteria. Typical receptors of Gram negative bacteria are the lipopolysaccharide, pili, flagella and other surface proteins. In Gram positive bacteria the peptidoglycan, teichoic and lipoteichoic acids as well as surface proteins are typical receptors for phages (Guttman *et al.*, 2005).

After the phage is irreversible attached to the host by binding to a second receptor, the DNA is transferred directly into the bacterial cell by using an enzymatic mechanism for penetrating the peptidoglycan and the inner membrane of the host (Molineux, 2001; Roos *et al.*, 2007). Once inside the cell the DNA has to be protected from host exonucleases and restriction enzymes. Common mechanisms for protection is the circularization of the nucleic acid, inhibition of host nucleases or using odd nucleotides like hydroxymethyldeoxyuridine (Arber & Dussoix, 1962; Dussoix & Arber, 1962; Kallen *et al.*, 1962).

Then the phage promotors are recognized by the host RNA polymerase and early genes will be transcribed. The gene products of these early genes are involved in

Table 1: Overview of phage families. Derived from (Ackermann, 2007). *Awaiting classification.

Shape	Nucleic acid	Familiy	Particulars	Example
Tailed	DNA	*Myoviridae*	tail contractile	T4
		Siphoviridae	tail long, nonconractile	λ
		Podoviridae	tail short	T7
Polyhedral	DNA	*Microviridae*	conspicuous capsomers	ϕX174
		Corticoviridae	complex capsid, lipids	PM2
		Tectiviridae	inner lipid vesicle, pseudotail	PRD1
		SH1	inner lipid vesicle	SH1
		STV1	turret-shaped protrusions	ST1V
	RNA	*Leviviridae*	poliovirus-like	MS2
		Cystoviridae	envelope, lipids	ϕ6
Filamentous	DNA	*Inoviridae*	a. long filaments	fd
			b. short rods	MLV1
		Lipothrixviridae	envelope, lipids	TTV1
		Rudiviridae	TMV-like	SIRV-1
Pleomorphic	DNA	*Plasmaviridae*	envelope, lipids, no capsid	L2
		Fuselloviridae	same, lemon shaped	SSV1
		Salterprovirus	same, lemon shaped	His1
		Guttaviridae	droplet shaped	SNDV
		*Ampullaviridae**	bottle shaped	ABV
		*Bicaudaviridae**	two tailed, growth cycle	ATV
		*Globuloviridae**	paramyxovirus like	PSV

the host takeover. Host proteases, restriction enzymes and host biosyntheses are inactivated before middle and late genes are transcribed. During the transcription of middle and late genes, new phage particles are synthesized and assembled before the bacterial cell bursts, induced by endolysins of the phage (Guttman *et al.*, 2005). This process describes the life cycle of a lytic phage (Fig. 1).

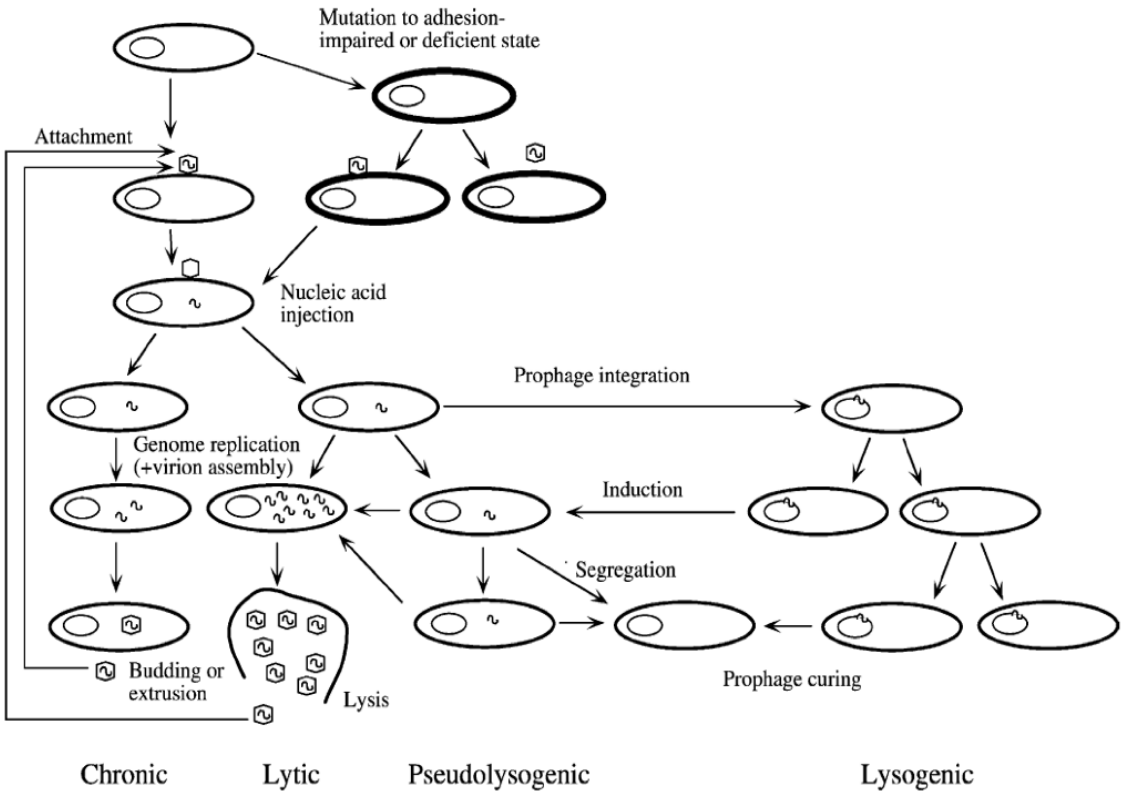


Figure 1: Types of viral life cycles (Weinbauer, 2004).

Lysogenic or temperate phages on the other hand can insert their genome into the host genome. The phage is now called prophage and can coexist within the host. Changes in the environment can lead to an induction of the prophage which will induce the lytic life cycle. However, two other life cycles have to be mentioned. Some phages can cause a chronic infection in bacteria. Thereby, the cell is infected

by a phage and releases constantly phage progeny from the host by budding or extrusion without lysing it. Moreover, some bacteria carry a plasmid-like prophage which is also called persistent infection or pseudolysogeny (Fig. 1). In this state phages multiply only in a fraction of the bacterial population (Weinbauer, 2004).

1.2 Importance of Bacteriophages Today

Since the discovery of bacteria specific viruses by William Twort (Twort, 1915) and by Felix d'Herelle (d'Herelle, 1917) phage research is without doubt an important part in biology. The research with bacteriophages resulted in important discoveries as the identification of the genetic code and the DNA as genetic material and depicts the first steps of molecular biology (Cairns *et al.*, 1966). Moreover, phages and phage derived products such as vectors, cosmids and promotors were important for the development of recombinant DNA techniques. Other phage derived products like integrases, DNA ligases and various other enzymes are an integral part of today's molecular biology (Summers, 2006).

After these great discoveries, phages were no longer in the focus of science. But recently a new interest in phages emerged due to genome research and the emerging antibiotic resistance of bacteria.

Genome research revealed that most bacteria carry lysogenic phages and that these so-called prophages play an important role on the host genome evolution (Canchaya *et al.*, 2003; Brüssow *et al.*, 2004). Also *P. aeruginosa* PAO1 carries three regions in the genome which contain prophages. Two of these loci contain the so-called pyocins which have an antimicrobial action. The region PA0610 to PA0648 is depicted as R and F pyocin and unlike other pyocins they resemble the phage tails of P2 and Lamda. The region PA0715-PA0729 has strong homologies to the phage Pf1. This prophage is integrated in a gene which encodes for the glycin tRNA (Hayashi & Nakayama, 2004).

More recently, new estimations about the diversity of phages and the impact of these viruses on ecosystems are highly discussed (Abedon, 2009; Rohwer, 2003; Vos *et al.*, 2009). Besides, the upcoming antibiotic resistance of pathogenic bacteria also

pushes the phages back into the spotlight. Researcher are forced to look for new alternatives to treat these bacteria. An important aspect plays the nearly forgotten phage therapy as a new "old" alternative to treat antibiotic resistant pathogens (Skurnik & Strauch, 2006).
Some of the mentioned aspects will be discussed in more detail in the following sections.

1.2.1 Global Diversity of Bacteriophages

Recent publications estimate about 6 million free living microbial species in the world (Curtis *et al.*, 2002). Moreover, it was shown that natural habitats like soil and water contain 10 - 100 times more phages than bacteria (Clokie & Kropinski, 2009). But the abundance of phages varies in different environments and is related to the abundance of the bacteria. Due to metagenome approaches it is estimated that there are 10^{31} viruses on earth, most of them are viruses infecting bacteria since bacteria are the most common prey items in the environment (Breitbart & Rohwer, 2005).
Phages are divided into families by morphology and nature of nucleic acid (1.1). Ackermann (2007) pointed out that 96 % of the investigated phages belong to the tailed phages and only the rest belong to the so-called pleomorphic, filamentous or polyhedral phages. That means that most of the phages belong to the order Caudovirales. There are three known phage families belonging to this order, *Myoviridae*, *Siphoviridae* and *Podoviridae*. They all exhibit dsDNA as nucleic acid (Tab. 1).

In December 2009 554 complete genome sequences of phages were available, among these sequences were 45 sequences of *Pseudomonas* specific phages (National Center for Biotechnology Information). In addition, sequences from metagenomic approaches of viruses were also published. Breitbart & Rohwer (2005) described that about 75 % of all sequenced genes did not match to any gene in databases, indicating that most of the viral diversity is uncharacterized today. Moreover, it was evaluated that there are about 100 million phage species in the world (Rohwer, 2003). On the other

hand it was shown that certain conserved genes of phages were found in different environments, suggesting that the local diversity is quite high but global diversity is limited and that phages are moving between habitats (Breitbart *et al.*, 2004). Also, Ceyssens *et al.* (2009) showed that the *Pseudomonas* infecting PB1-like phages are widespreaded in nature and very conserved. This supports the theory that global virus diversity is limited.
Still, the genetic potential behind the phages and their impact on their hosts, the bacteria, and hence to the environment should be immense and will be discussed in the next section.

1.2.2 Influence of the Phage on the Bacterium and the Environment

Phages are an important genetic element in the evolution of bacteria since they can transfer bacterial DNA from one to an other bacterium. This process is called transduction which is a form of lateral gene transfer and is not exclusively species specific, since some phages can infect more than one species. During an infection process it happens that bacterial DNA is accidently packed into the new virion particle which is called generalized transduction. Specialized tranduction on the other hand happens when a prophage removes itself incorrectly from the host DNA and a part of the host DNA will be transferred via the progeny phages (Canchaya *et al.*, 2003).

Some phage genes encode for virulence factors which can increase the virulence of a bacterium or convert their bacterial host from a non-pathogenic to a pathogenic strain. This process is called lysogenic conversion (Waldor & Friedman, 2005). For instance, *Corynebacterium diphtheriae* infects the upper respiratory tract and causes inflammation and necrosis. The dreaded diphtheria toxin is only produced when the bacterium carries the temperate phage β which encodes this protein (Freeman, 1951). Moreover, some diarrhoegenic *E. coli* strains produce a group of related toxins, the Shiga toxins, whose genes are part of the genome of lamboid prophages (Herold *et al.*, 2004). The phage ϕCTX was isolated from cytotoxin producing

P. aeruginosa strains (Nakayama *et al.*, 1999). The gene coding for the cytotoxin is located on the genome of this phage. The cytotoxin makes *P. aeruginosa* more virulent since the active cytotoxin makes holes in the cytoplasmic membrane of eukaryotes. Other examples of lysogenic conversion comprise effects on proteins altering antigenicity, effector proteins involved in invasion, different virulence related enzymes and also effects on the adhesion of the bacterium to the eukaryotic host (Brüssow *et al.*, 2004).
Moreover, it was also shown that phages have an impact on the phenotypic variation of *P. aeruginosa*. Rice *et al.* (2009) demonstrated that the filamentous prophage Pf4 is essential for several stages of the biofilm life cycle. Pf4 deficient mutants did not undergo cell death and are less virulent. Genes encoding for prophages are also the most upregulated genes during biofilm formation (Whiteley *et al.*, 2001). But not only biofilm variation also the emergence of small colony forming variants (SCVs) seem to be dependent on the prophage (Webb *et al.*, 2004). In addition, the pili-dependent RNA phage PP7 drives morphological diversification in *P. aeruginosa*, SCVs coexists with large colonies in a bacterial population when the phage is present.

Since all the depicted examples affect the bacterium itself the process of phages killing bacteria have also a more global impact on ecosystems. Heldal & Bratbak (1991) estimated that phages might kill 4-24 % of the bacterial population per hour in aquatic systems and represents an important factor limiting bacterial populations. This shows that the viral community of an environment has a great impact on microbial food web processes and on biogeochemical cycles (Weinbauer, 2004). Bacteria are important for decomposition of other organisms as well as they consume, produce and store nutrients. Lysis of bacteria makes nutrients available but has also an impact on the biogeochemical pathways where bacteria are involved (Abedon, 2006). It was published that viruses for example limit the primary productivity of photosythetic bacteria in the ocean (Suttle, 1994). Moreover, phages are an important biotic carbon pool and due to their high protein and DNA content, phages may also contribute significantly to the biotic nitrogen and phosphorus pool (Weinbauer, 2004).

Also, the impact of phages on the bacteria of the human or animal flora were investigated. Most of published data refer to the occurence of bacteriophages in the gastrointestinal contents of animals. It was shown that phage populations in intestinal and rumenal microbial systems vary between species and individuals (Letarov & Kulikov, 2009).
However, it was also demonstrated that phages were present in the lung of patients with cystic fibrosis and active against *P. aeruginosa* (Brockhurst *et al.*, 2005). Atterbury *et al.* (2005) could show that the population of *Campylobacter jejuni* in the intestine of chickens negatively correlates with the phage population. And it was later presented by Scott *et al.* (2007) that these phages select for hosts with large-scale genomic rearrangements that occur via lateral gene transfer.

1.2.3 Phage Therapy

Phage therapy has a long history. It started directly after the discovery of phages. D'Herelle was convinced about the therapeutical potential of the phages due to their specificity to bacteria and nontoxicity to animals and plants. In his first attempts he treated dysentery patients successfully (Summers, 2001). Later in 1921 Joseph Maisin used phages to treat staphylococci induced skin diseases (Sulakvelidze *et al.*, 2001). However, after the discovery of the first antibiotics in the western countries phage therapy was forgotten, also because of the poorly investigated phage biology. Only some countries in the former Soviet Union, especially the Elivia Institute in Georgia, continued developing phages as a therapeutical agent (Summers, 2001).
Today after the worldwide emergence of antibiotic resistant pathogenic bacteria phage therapy became again a possibility to treat bacterial infections. Advantages over antibiotics are the specificity to certain bacteria and that phages can be isolated and investigated rapidly. The upcoming of phage resistant bacteria can be easily prevented by using phage cocktails (Sulakvelidze *et al.*, 2001). Phages are nontoxic and can be applied to surface infections without adverse effects as described in Rhoads *et al.* (2009). On the other hand, the application of phages orally or

intravenously is still highly discussed, because little is known about the reaction of the immune system to the phages, the clearance of the phages in the body and the actual effects on the infection.

But not only whole phages are used today. Some attempts showing that isolated endolysins, the enzymes which helps the phage to free the progeny phages after a complete lifecycle, can be used to treat infections of Gram positive bacteria (Fischetti, 2005). Jado *et al.* (2003) used a phage encoded murein hydrolase to treat *Streptococcus pneumoniae* infected mice. They suggest that phage lysins protect animals from bacterial infections due to their results that the used murein hydrolase rescued the infected mice in their study.

1.3 *Pseudomonas aeruginosa*

1.3.1 The Bacterium *Pseudomonas aeruginosa*

Pseudomonas aeruginosa belongs to the group of free-living γ-proteo bacteria and is ubiquitous in water and soil environments at a low frequency. It is a polar flagellated rod (Fig. 2) with a high GC content of 68 % (Palleroni, 1992).

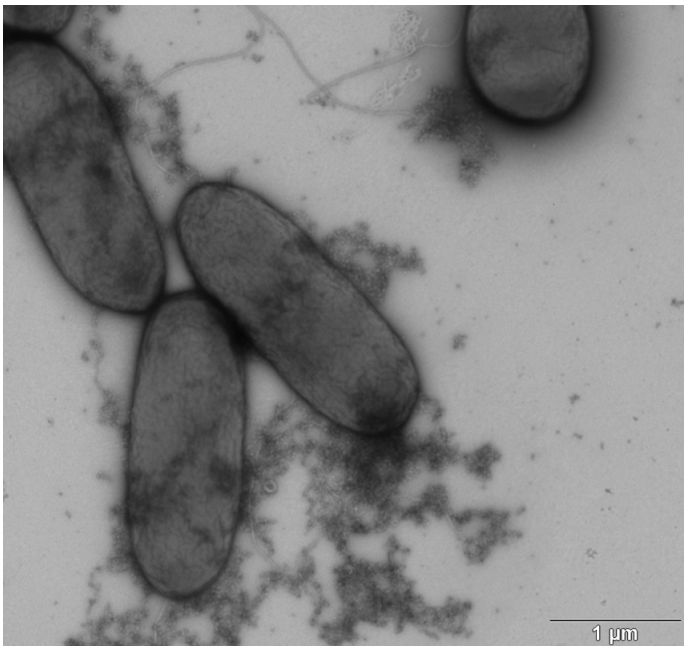


Figure 2: Electron microscopical picture of *Pseudomonas aeruginosa*. It has a length of approximately 2 μm. The long thin structures in this picture are flagella whereas the small round structures are membrane vesicles.

P. aeruginosa is able to produce several pigments like the green-fluorescent pigment pyoverdin or the virulence related pigment pyocyanin which give cultures a characteristic green-blue color (Palleroni, 1992).
The genome of *P. aeruginosa* PAO1 was published by Stover *et al.* (2000) and depicts a large genome with a size of 6.3 Mbp and 5,570 predicted open reading frames (ORF). 9.4 % of the ORFs encode for regulatory genes and two-component systems. This leads to the suggestion that this bacterium is able to respond to

different environments and changes within these environments. *P. aeruginosa* has a high nutrient versatility and about 300 cytoplasmic transport systems (Stover *et al.*, 2000). The genome size of other sequenced *P. aeruginosa* strains varies in a range from 5 - 7 Mbp suggesting adaptations to different environmental niches (Schmidt *et al.*, 1996).

It is facultative anaerob, prefers oxygen as the terminal electron acceptor and is able to degrade various carbon sources, e.g. sugars, fatty acids, alcohols, glycols, aromatic compounds, amines and also amino acids (Palleroni, 1992).

In contrast to the *Enterobacteriaceae* which degrade glucose via the Embden Meyerhoff Parnas pathway, *P. aeruginosa* uses the Entner Doudoroff pathway (KDPG-pathway) although it favours the degradation of organic acids (Palleroni, 1992).

Under anaerobic conditions *P. aeruginosa* performs denitrification using nitrate or nitrite as alternative electron acceptors. In the absence of a terminal electron acceptor *P. aeruginosa* fermentates the amino acid arginine (Wauven *et al.*, 1984) as well as pyruvate (Eschbach *et al.*, 2004). Pyruvate fermentation supports only long-term survival under anaerobic conditions but no growth.

Moreover, *P. aeruginosa* is able to form biofilms. Biofilms are structured communities of bacteria adherent to a surface and covered in a polymeric matrix (Costerton *et al.*, 1999). Biofilms are often involved in infections (see also next section). Due to the nature of the biofilm antibodies of the host immune system, antimicrobials and phages are nearly ineffective (Costerton *et al.*, 1999).

1.3.2 Pathogenicity and Antimicrobial Resistance of *Pseudomonas aeruginosa*

Additional to its high nutrient versatility and its appearance in different environments *P. aeruginosa* is also well known as an opportunistic pathogen for plants, animals and humans using the same spectrum of virulence factors in all its hosts (Rahme *et al.*, 1995). This bacterium is related to many infections and diseases in humans mainly in predisposed immunocompromised patients due to chemotherapy, transplantations and HIV-infections (VanDelden & Iglewski, 1998). It can cause

urinary tract, respiratory tract, ear, eye and burn wound infections. Respiratory tract infections caused by *P. aeruginosa* are dreaded in patients suffering from Cystic Fibrosis (Mucoviscidose) since these infections often cause the total loss of lung function. Moreover, Tümmler & Kiewitz (1999) pointed out that *P. aeruginosa* is an important nosocomial germ which belongs to the five most common pathogens isolated in hospital-wide surveillance.

P. aeruginosa exhibits various resistance mechanisms towards antibiotics. The most important mechanisms for the well known and dreaded innate antimicrobial resistance are the low outer membrane permeability and the expression of several multidrug efflux pumps (Schweizer, 2003). The permeability of the outer membrane in *P. aeruginosa* is even lower than in *Escherichia coli* (Yoshimura & Nikaido, 1982). Chromosomally-encoded efflux systems transport not only antibiotics but also structurally distinct classes of biocides, dyes, detergents and even quorum sensing molecules across the membranes (Schweizer, 2003). The asymmetric bilayer of the outer membrane with its variable lipopolysaccharide (LPS) serves as an effective barrier against the rapid penetration of lipophilic antimicrobials (Nikaido, 1994; Poole, 2002) and completes the innate resistance of this clinical important species. Moreover, the biofilm lifestyle of *P. aeruginosa* serves also as a mechanism of resistance, since the biofilm restricts the diffusion of the antibiotic and also reduces the growth rate, which makes most of the antimicrobial agents less effective (Drenkard, 2003).

P. aeruginosa expresses five different multidrug efflux systems (Mex) which promote multidrug resistance due to hyperexpression of the efflux genes (Schweizer, 2003). These efflux pumps belong to the RND (resistance nodulation devision) familiy of efflux pumps and have a tripartite system. They are driven by the proton motive force and consist of a transporter protein which is located in the inner membrane, an outer membrane protein and a accesory protein which is located in the periplasm (Piddock, 2006).

It was shown that treatment of *P. aeruginosa* with antibiotics leads to mutations in the gene *mexR* (Ziha-Zarifi *et al.*, 1999). MexR is the negative regulator of the *mexABoprM* efflux operon and belongs to the MarR family of transcriptional regulators

(Poole *et al.*, 1996). Besides MexR, this efflux pump is also regulated by two other negative regulators, NalC (Cao *et al.*, 2004) and NalD (Morita *et al.*, 2006). Moreover, it is suggested that this efflux pump has also a role in the pathogenesis of *P. aeruginosa* since strains lacking this efflux pump were not able to infect leukocyte deficient mice in contrast to the wild type (Hirakata *et al.*, 2002).
Recently, a proposed model for the mechanism of the MexR-regulated antibiotic resistance was published. Chen *et al.* (2008) showed that the MexR regulator exhibits two redox-active cystein residues which enables the regulator to sense oxidative stress in the cell. This oxidative stress in the cell could be triggered by antibiotics which stimulate the hydroxyl radical formation via the Fenton reaction (Kohanski *et al.*, 2007). Moreover, oxidative stress induced by antibiotics or the leukocytes during infection might also be involved in the development of resistance due to oxidative DNA damage in the cell (Mandsberg *et al.*, 2009).

1.3.3 The Lipopolysaccharide (LPS) of *Pseudomonas aeruginosa*

The LPS of *P. aeruginosa* is an important virulence factor on the outer leaflet of the outer membrane. The LPS structure of *P. aeruginosa* is similar to the LPS of other Gram negative bacteria and consists of three domains: the lipid A, the core oligosaccharide and the O polysaccharides. It is a physical barrier, can mediate direct interaction with the host and, as an endotoxin, damages host tissues (King *et al.*, 2009).
The lipid A anchors the LPS to the outer membrane. It consists of fatty acids which are attached to doubly-phosphorylated diglucosamines. Most strains show a penta- or hexa-acylated form (King *et al.*, 2009). The lipid A is responsible for the toxicity of Gram negative bacteria because this part of the LPS is recognized by the TLR4 (toll-like receptor 4) receptor complex in humans which activates the immune system. But the lipid A of *P. aeruginosa* is significantly less toxic than the lipid A of enteric bacteria like *E. coli* due to the length and number of acyl chains (Bäckhed *et al.*, 2003).
The core oligosaccharide is directly attached to the lipid A and can be divided into

the inner and outer core. The inner core consists of two 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) and two heptose residues which serve as the phosphorylation site (Fig. 3). One Kdo is linked to one glucosamine of the lipid A. The outer core has three different sugar types: glucose, rhamnose and galactoseamine. *P. aeruginosa* can produce two glycoforms of the LPS simultaneously (Fig. 3). Both glycoforms share the same inner core structures but the glycoform 1 is never substituted by O-antigen (King *et al.*, 2009).

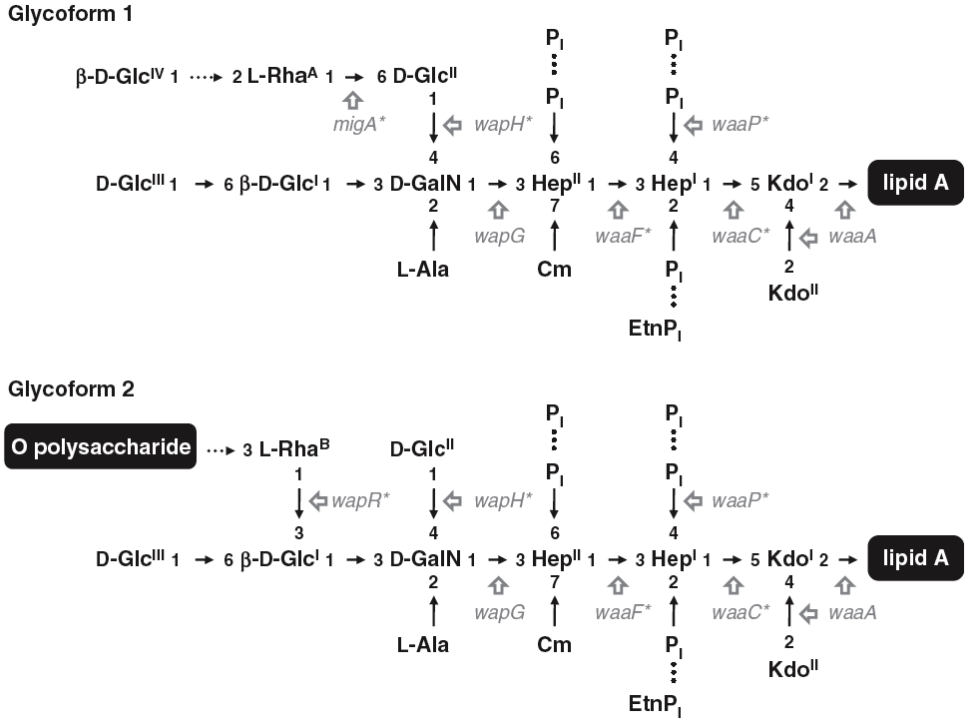


Figure 3: LPS structure and the comparison of the two glycoforms of the LPS core (King *et al.*, 2009). Genes involved or putatively involved (*) in enzymatic steps are indicated. Ala, alanine; Cm, carbamoyl; Etn, ethanolamine; GalN, 2-amino-2-deoxy-galactose; Glc, glucose; Hep, L-glycero-D-manno-heptose; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; Rha, rhamnose.

Due to their O specific antigen *P. aeruginosa* strains can be divided into serogroups. Currently, there are 20 different serogroups of *P. aeruginosa* (Liu & Wang, 1990). The O specific antigen itself consists of sugar repeats with different constitution. Moreover, Rivera *et al.* (1992) pointet out that many *P. aeruginosa* strains carry a second LPS species which is called common O polysaccharide and is a rhamnan homopolymer. In some publications the O specific antigen is called B-band LPS whereas the common O polysaccharide is described as A-band LPS.

There are several examples that the LPS of *P. aeruginosa* could have an impact on the resistance of this bacterium. Mutations which lead to changes in the LPS profile of *P. aeruginosa* when treated with antibiotics like ciprofloxacin (Legakis *et al.*, 1989), gentamicin (Bryan *et al.*, 1984; Shearer & Legakis, 1985) and also carbenicillin (Godfrey *et al.*, 1984; Shearer & Legakis, 1985) were reported. These studies have in common that the alteration of the LPS contributes to the antibiotic resistance, suggesting that the altered LPS may impair the uptake of the antimicrobial compound.

Moreover, it was also shown that the phosphate residues of the core LPS play an important role in the intrinsic resistance of *P. aeruginosa*. Walsh *et al.* (2000) showed that additional phosphate residues can lead to an increase of antimicrobial resistance. Also, Schurek *et al.* (2008) showed that aminoglycoside resistance in *P. aeruginosa* is a product of several variable mechanisms and that LPS mutants had a reduced susceptibility to tobramicin.

1.4 Aims of the Study

The aim of this study was divided into two parts. In the first part *Pseudomonas aeruginosa* specific phages had to be isolated and characterized. In this context, the host receptor, the host spectrum, the burst size and the duration of the infection cycle were of interest. Moreover, DNA sequence analysis of the corresponding genomes and comparison to known phage genomes were envisaged.

Successful isolated lipopolysaccaride specific phages should be used in a second part of this thesis as a screening tool for the investigation and isolation of *P. aeruginosa* strains with truncated LPS. We were interested if the truncated LPS contributes to antibiotic tolerance of *P. aeruginosa* and if bacteriophages are suitable tools for these investigations. To monitor the emergence of *P. aeruginosa* strains with an truncated LPS during antibiotic stress, a phage based *in vitro* system had to be developed. Using this phage based *in vitro* system, the exact role of the truncated LPS in antibiotic resistance as well as the mutation causing this phenotype should be elucidated.

2 Materials and Methods

2.1 Instruments and Chemicals

2.1.1 Instruments

Agarose gel electrophoresis	Agagel (Biometra)
Agarose gel documentation	GelDoc (Bio-Rad)
Autoclave	EL3850 (Systec)
Biological Safety Cabinet	HeraSafe (Heraeus)
Centrifuges	Minispin (Eppendorf)
	Biofuge fresco (Heraeus)
	Megafuge 1.0 R (Heraeus)
DNA sequencing	Genetic Analyzer ABI Prism^{TM} (Applied Biosystems)
Incubator	Fine Line (Heraeus)
	Thermo Shaker PST-60 HL-4 (Lab4You)
	Aquatron (Infors)
pH determination	Microprocessor pH Meter 211
Photometer	Ultrospec (Amersham Biosciences)
SDS-PAGE system	MiniProtean II (Bio-Rad)
Spectrophotometer	Nanodrop ND-1000-UV/VIS (PEQLAB)
Thermocycler	Tpersonal (Biometra)
	Tgradient (Biometra)
Thermomixer	Thermomixer compact (Eppendorf)
Vortex	Vortex Genie 2 (Scientific Industries)
Water purification	MilliQ System
Weighing machine	572 (Kern & Sohn)
	ALJ 160-4NM (Kern & Sohn)

2.1.2 Chemicals and Kits

Chemicals and reagents not specifically listed here were purchased from the following manufacturers: Amersham Biosciences, Fluka, Merck, Roth, Sigma-Aldrich, Riedel-de-Häen.

5-aminolevulinic acid	Sigma
Benzonase Nuclease	Novagen
GeneRuler^{TM} DNA Ladder Mix	MBI Fermentas
Oligonucleotides	Metabion
Primer	Metabion
Proteinase K	Sigma-Aldrich
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Restriction Endonucleases	New England Biolabs $_{INC}$
SAP (Shrimp Alkaline Phosphatase)	MBI Fermentas
T4 DNA ligase	New England Biolabs $_{INC}$
Taq DNA Polymerase	GE Healthcare

2.2 Bacteria, Phages and Plasmids

All phages, bacterial strains and plasmids used for this work are listed in Table 2, Table 3, Table 4 and Table 5.

Table 2: List of Bacteriophages

Plasmid	Description	Reference
JG004	*P. aeruginosa* PAO1 bacteriophage	This work
JG024	*P. aeruginosa* PA14 bacteriophage	This work
SZ06	*P. aeruginosa* PAO1 TM5 bacteriophage	This work

Table 3: List of Plasmids

Plasmid	Description	Reference
pEX18Ap	Ap^r; *oriT*$^+$ *sacB*$^+$	Hoang *et al.* (1998)
pFLP2	Ap^r; Flp coding plasmid	Hoang *et al.* (1998)
pPS858	Ap^r; Gm^r	Hoang *et al.* (1998)
pJG002	Ap^r Gm^r; pEX18Ap with 501 bp upstream *mexR*, Gm^r-gfp fragment from pPS858, and 432 bp downstream of the coding region of *mexR* between *Sac*I and *Hind*III	This work
pJG005	Ap^r Gm^r; pEX18Ap with 386 bp upstream PA5001, Gm^r-gfp fragment from pPS858, and 500 bp downstream of the coding region of PA5001 between *Sac*I and *Hind*III	This work
pUCP20T	mobilizable *E. coli* - *P. aeruginosa* shuttle vector; Cb^r	Schweizer *et al.* (1996)
pJG004	pUCP20T with coding region of PA5001 between *Xba*I and *Kpn*; Cb^r	this work
pBT20	Mini-TnM delivery vector, Ap^rGm^r	Kulasekara *et al.* (2005)
pLAFR3	Tc^r, broad host range cosmid	Staskawicz *et al.* (1987)

Table 4: List of *Escherichia coli* strains

Strain	Description	Reference
DH10B	F- *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) 80 *lacZ* ΔM15 Δ*lacX*74 *deoR recA1 endA1 araD* Δ(*ara, leu*)	Invitrogen
ST18	*pro thi hsdR*$^+$ Tp^r Sm^r; chromosome::RP4-2 Tc::Mu-Kan::Tn7/λ*pir* Δ*hemA*	Thoma & Schobert (2009)
S17λ*pir*	*pro thi hsdR*$^+$ Tp^r Sm^r; chromosome::RP4-2 Tc::Mu-Kan::Tn7/λ*pir*	de Lorenzo & Timmis (1994)

Table 5: List of *Pseudomonas aeruginosa* strains

Strain	Description	Reference
PAO1	wild type	Dunn & Holloway (1971)
PA14	wild type	Rahme *et al.* (1995)
JG01	PAO1Δ*algC*	Julia Garbe, unpublished
TM5	PAO1 transposon mutant	This work
TM6	PAO1 transposon mutant	This work
PAO1 DK	wild type	Klausen *et al.* (2003)
PAO1 DK *pilA*	PAO1 DK Δ*pilA*	Klausen *et al.* (2003)
PAO1 DK *fliM*	PAO1 DK Δ*fliM*	Klausen *et al.* (2003)
JG09	PAO1Δ*mexR*	This work
JG94	PAO1Δ*PA5001*	This work
JG95	PAO1Δ*mexR* ΔPA5001	This work

Strain	Description	Reference
GH06 41910	clinical isolate	Michael Hogardt, München
GH06 54733-2	clinical isolate	Michael Hogardt, München
GH06 21466	clinical isolate	Michael Hogardt, München
GH06 25135	clinical isolate	Michael Hogardt, München
GH06 20934	clinical isolate	Michael Hogardt, München
BT2	clinical CF isolate	MHH
BT72	clinical CF isolate	MHH
BT73	clinical CF isolate	MHH
RN3	clinical CF isolate	MHH
NN84	clinical CF isolate	MHH
PACF15	clinical CF isolate	Gerd Döring, Tübingen
PACF21	clinical CF isolate	Gerd Döring, Tübingen
PAKL1	clinical CF isolate	Gerd Döring, Tübingen
PAKL4	clinical CF isolate	Gerd Döring, Tübingen
PACF60	clinical CF isolate	Gerd Döring, Tübingen
PACF61	clinical CF isolate	Gerd Döring, Tübingen
PACF62	clinical CF isolate	Gerd Döring, Tübingen
PACF63	clinical CF isolate	Gerd Döring, Tübingen
MH 18	clinical urinary tract isolate	Michael Hogardt, München
MH 19	clinical urinary tract isolate	Michael Hogardt, München
MH 26	clinical urinary tract isolate	Michael Hogardt, München
MH 29	clinical urinary tract isolate	Michael Hogardt, München

2.3 Buffers

Frequently used buffers:

Tris Acetate EDTA (TAE) buffer:	40	mM Tris-acetate
	1	mM EDTA
		in dH_2O, pH 8.0
Phosphate buffered Saline (PBS) buffer:	137	mM NaCl
	2.7	mM KCl
	10	mM Na_2HPO_4
	2	mM KH_2PO_4
		in dH_2O
SM buffer:	100	mM NaCl
	8	mM $MgSO_4$
	50	mM Tris-HCl
		in dH_2O, pH 7.5

2.4 Media and Media Additives

2.4.1 Growth Media

Luria-Bertani (LB) medium was used as a standard komplex medium for growth of *P. aeruginosa* and *E. coli*. The composition is based on the protocoll of Sambrook (1989)

LB medium:	1	% (w/v) trypton
	0.5	% (w/v) yeast extract
	0.5	% (w/v) NaCl
		in dH_2O

For LB agar plates 15 g/l agar was added to the medium.

2.4.2 Media Additives

Concentrated stock solutions of media additives like sucrose, ALA and antibiotics were prepared and sterilized by filtration (pore width 0.2 μm). Additives were dissolved in MilliQ water, except for chloramphenicol and tetracycline. Chloramphenicol was dissolved in 100 % ethanol and tetracycline as well as levofloxacin in 50 % ethanol. Concentrations for stock solutions and final concentrations are listed in Table 6. MIC indicates that the antibiotics were used in different concentrations as described in 2.5.8 (MIC: minimal inhibitory concentration).

Table 6: Media Additives

Substance	Stock solution	Concentration for *P. aeruginosa*	Concentration for *E. coli*
Carbenicillin	100 mg/ml	500 μg/ml/MIC	100 μg/ml
Gentamicin	30 mg/ml	80 μg/ml	10 μg/ml
Chloramphenicol	100 mg/ml	-	10 μg/ml
Tetracycline	5 mg/ml	80 μg/ml	5 μg/ml
Levofloxacin	20 mg/ml	MIC	-
Nalidixic Acid	5 mg/ml	MIC	-
ALA	50 mg/ml	-	50 μg/ml

2.5 Microbiological Techniques

2.5.1 Sterilization

All media were autoclaved at 121 °C and 1 bar positive pressure for 20 min. Other substances and solutions were either autoclaved or, if temperature sensitive, sterilized by filtration (pore width 0.2 μm).

2.5.2 General Growth Condition

Aerobic cultured bacteria were incubated in Erlenmeyer flasks at 37 ℃ and 200 rpm (Aquatron). The incubation time depended on the experiment and is indicated. *E. coli* ST18 was always grown in the presence of 50 μg/ml aminolevulinic acid (ALA) since this strain is *hemA* deficient and can not synthesize ALA.

2.5.3 Determination of Cell Density

The cell density of liquid cultures was determined by measuring the optical density (OD) at a wavelength of 578 nm. An OD_{578} of 1.0 corresponds to approximately 1 x 10^9 cells/ml.

2.5.4 Determination of Colony Forming Units

The colony forming unit was determined by diluting an aseptic taken sample in PBS buffer. Appropiate dilutions were plated on LB agar plates and incubated for 16 to 24 h at 37 ℃. Afterwards the visible colonies were counted and the colony forming unit per ml was calculated.

2.5.5 Storage of Bacterial Strains

Glycerol stocks were prepared for long-term storage. For this 850 μl of overnight cultures were mixed with 150 μl of sterile glycerol (15 %). Stocks were frozen and kept at -80 ℃.

2.5.6 Determination of Minimal Inhibitory Concentrations (MIC)

The minimal inhibitory concentration (MIC) was determined in 96 well plates. 10^5 cells per ml of an overnight culture were inoculated in LB with appropriate concentrations of antibiotics, H_2O_2 or menadione. The overnight culture was grown in the same medium at 37 ℃ and 200 rpm for 12 h. 96 well plates were then incubated at 37 ℃ in a microplate incubator for 20 h. MICs were estimated as the lowest concentration of antibiotics which inhibited growth.

2.5.7 Antibiotic Susceptibility Test

Antibiotic susceptibility tests stribes ATB^{TM} PSE I were purchased from Bio-Mérieux and used according to manufacturer's instructions. Each strain were tested in dublicate. Antibiotic susceptibility was categorized as sensitive (no growth), intermediate (growth in the presence of low antibiotic concentrations) and resistant (growth in the presence of high antibiotic concentrations).

2.5.8 Menadione Survival Assay

To test the tolerance to oxidative stress, menadione was used as a superoxide radical producer. Cells of an overnight culture were pelleted and resuspended in PBS buffer to an OD_{578} of 0.1. Menadione sodium bisulfite (Sigma-Aldrich) was added in various concentrations ranging from 2.5 mg/ml to 10 mg/ml and the cells were incubated at 37 ℃ for 4 h. After incubation aseptic taken samples were diluted in PBS buffer and plated on LB agar plates. Before bacterial enumeration and determining the CFU (2.5.4) the LB agar plates were incubated for 12 to 24 h at 37 ℃.

2.5.9 Phage Isolation

Phages were isolated from sewage following a simple enrichment procedure. Samples from sewage were centrifuged for 5 min at 4100 x g (Biofuge fresco). Ten ml of the supernatant were mixed with 5 ml of an *P. aeruginosa* overnight culture and incubated in 50 ml LB broth at room temperature. After an incubation of 48 h the cells were sedimented by centrifugation at 4100 x g (Biofuge fresco) for 10 min and the supernatant was transferred to a clean tube. To kill the remaining bacteria, several drops of chloroform were added to the supernatant and the mixture was mixed for 30 s. To separate the phages, appropriate dilutions of the phage lysate were spotted onto bacterial lawns of top-agar plates. Top-agar plates were produced by adding approximately 5 x 10^8 cells/ml of *P. aeruginosa* from an overnight LB broth to 3.5 ml of LB top agar (0.75 %). The inoculated top-agar was overlaid on an LB agar plate and allowed to solidify. After incubation at 37 ℃ for 10 to 16 h zones of lysis were recognized. Single plaques, derived from a single phage, were

separated by stinging with a pipette tip into the plaque followed by resuspending the phages in SM buffer. The resulting phage lysate was stored at 4 ℃.

2.5.10 Propagation of Phages

To propagate phages top-agar plates were prepared as described in 2.5.9. Additionally, 100 μl of a phage lysate was added to the top-agar. After 12 to 16 h of incubation at 37 ℃ lysis of the bacteria were recognized and the plates were overlaid with 10 ml of SM buffer. This was followed by 4 h incubation at 4 ℃ to dissolve the phages from the top-agar. The SM buffer enriched with the phages was removed from the plates and centrifuged for 10 min at 4 ℃ and 4100 x g (Biofuge fresco) to pellet the remaining bacteria. The supernatant was sterilized by filtration (pore width 0.2 μm) and the phage titer of the phage stock was measured.

2.5.11 Determination of Phage titer

The phage titer was determined by diluting a phage solution in SM buffer. Appropiate dilutions were spotted on a top-agar plate (2.5.9) with the appropiate host. After incubation for 12 to 16 h at 37 ℃ the plaques derived from single phages were counted and the plaque forming unit per ml was calculated.

2.5.12 Storage of Phages

For long-term storage of phages glycerol stocks were prepared. Therefore, 850 μl of a phage stock (2.5.10) was mixed with 150 μl of sterile glycerol (15 %). Stocks were frozen and kept at -80 ℃.

2.5.13 Phage Host Range Determination

To determine the phage host range, top-agar plates (2.5.9) with the potential host lawn were prepared. Ten μl of a phage stock solution were spotted on the top-agar plate and incubated at 37 ℃ for 12 to 16 h. After incubation, the appearance of the lysis zones at the site where the phage suspension was added, were examined.

Each phage was tested against each bacterial strain in triplicate in independent experiments. The lysis was catergorized as clear (+), turbid (0) and no reaction (-).

2.5.14 Electron Microscopy

Electron microscopy was performed by Dr. Manfred Rohde from the Helmholtz Centre for Infection Research (HZI). A phagetiter of 10^8 phages/ml was used.

2.5.15 Phage Growth Characteristics

To determine phage growth characteristics like the burst size and the duration of the infection cycle, single step growth experiments were performed as described in Pajunen *et al.* (2000). *P. aeruginosa* was grown aerobically in 20 ml LB medium till exponential growth phase (2.5.2). After the bacteria reached an OD_{578} of 0.3 an amount of 10^9 phages were added to the culture. Samples for OD measurement (2.5.3) to follow bacterial growth and samples for the determination of phage enumeration were taken aseptic at different time points after infection (0 min, 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 40 min, 60 min). To determine the phagetiter as described in 2.5.11 100 μl of an aseptic taken sample were centrifuged to pellet the bacteria in the sample and then diluted in SM buffer.

Burst Size

The burst size indicates the average number of phage progeny per infected cell at the end of one infection cycle. To calculate the burst size, the number of phages at the plateau of the sigmoidal curve was divided by the number of phages before the exponential growth begins (Carlson, 2005).

Duration of Infection Cycle

The duration of the infection cycle is described by the latent phase of the phage. In a single step growth curve at the end of the latent phase the amount of phages suddenly rises which describes the endpoint of the infection cycle because the progeny phages are released at this time point.

2.5.16 Phage Based *in vitro* System to Screen for LPS Variants

Screening of the transposon mutants is described in 2.6.7. To determine whether the LPS alterations in the *mexR* deletion mutant occur during antibiotic exposure the following method was used. An overnight culture of JG09 with and without carbenicillin (200 μg/ml) was serially inoculated over a period of 6 days. Each day 1 x 10^8 cells were incubated with a tenfold excess of bacteriophage JG004 for 1 h at 37 ℃. After incubation the colony forming units (2.5.4) of the phage resistant cells were determined. The colony forming unit of the cultures without exposure to the bacteriophage JG004 was also determined to calculate the exact number of phage resistant cells (2.5.4). The naturally occurence of phage JG004 resistant bacteria was determined as below 0.012 %. To determine this rate, the wild type PAO1 was incubated with the phage JG004 as described above in LB without antibiotics. The result indicate that phage JG004 is an applicable tool to screen for LPS alterations. To induce or prevent oxidative stress we used LB medium without bivalent ions using Chelex® 100 resin (Bio-Rad) following the manufactures instructions. Ferrous iron contributes to the Fenton reaction and therefore to hydroxyl radical formation whereas medium without ferrous iron prevents this formation. Trace metals (1 ml/l), magnesiumchlorid (1 mM) and calciumchlorid (100 μM) were added to the medium after Chelex treatment to permit growth of the bacteria. To induce oxidative stress by enforcing the Fenton reaction, 1 mM ferrous sulfate was added to the medium.

Trace metals:	200	mg $CaSO_4$ * 2 H_2O
	200	mg $FeSO_4$ * 7 H_2O
	20	mg $MnSO_4$ * H_2O
	20	mg $CuSO_4$ * 5 H_2O
	20	mg $ZnSO_4$ * 7 H_2O
	10	mg $NaMoO_4$ * H_2O
	5	mg H_3BO_3
		in 1 l dH_2O

2.5.17 Reactive Oxygen Species (ROS) Assay of *P. aeruginosa*

To determine the reactive oxygen species in single cells a culture of *P. aeruginosa* was grown aerobically in LB at 37 ℃ till early exponential phase. The growth was monitored by measuring the optical density (2.5.3). After the culture reached an OD_{578} of 0.4 antibiotics like carbenicllin (200 μg/ml) and gentamicin (5 μg/ml) or as a control the superoxid radical producer menadione (10 mg/ml) were added and followed by an incubation of 1h at 37 ℃. Finally the cells were diluted in PBS to an OD 0.5 and incubated with the flourescence dye H_2DCFDA (Invitrogen) according to manufacturer‘s instructions. The resulting flourescence of single cells was measured by flourescence activated cell sorting.

2.6 Molecular Biology Techniques

2.6.1 Polymerase Chain Reaction

The colony PCR (Polymerase chain reaction) technique was used to amplify genes directly from the *P. aeruginosa* genome. As DNA template a single *P. aeruginosa* colony from an agar plate was isolated, resuspended in sterile destilled water and mixed. *Taq* DNA polymerase (GE healthcare) was used for all reactions.
For a 50 μl PCR reaction 1 μl of the DNA template, 5 μl 10 x PCR-buffer, 10 pmol Primer, 200 μm dNTPs, 0.5 Units *Taq* DNA polymerase and 3 % (v/v) DMSO was mixed. The first denaturation step at 95 ℃ for 5 min was followed by a cycle of denaturation, primer annealing and elongation. This cycle was completed 30 times and the syntheses was finished after 5 min at 72 ℃. The denaturation at 95 ℃ for 1 min and the elongation temperature of 72 ℃ remained unchanged. The elongation time was chosen according to the length of the PCR fragment to be amplified. *Taq* DNA polymerase used in this work synthesizes approximately 1 kb in 1 min. The annealing temperature depended on oligonucleotide length and G+C content and was determined in a gradient cycler.
All PCR products were purified using the QIAquick PCR Purification Kit according to manufacturer‘s instructions.

2.6.2 Electrophoretic Separation of DNA

For the analytical separation of DNA fragments, 1 % (w/v in TAE buffer) agarose gels were prepared. Prior to use the DNA fragments were mixed with loading dye and the GeneRuler^{TM} DNA Ladder Mix was used as a size standard according to the manufacturer's instructions. A voltage of 100 V was applied to the loaded gels. After electrophoresis the gels were incubated in an ethidium bromide solution (0.1 % (v/v)) for 10-20 min to visualize the seperated DNA fragments under UV light (λ = 312 nm). If extraction of the DNA fragments was required the extraction was carried out using the QIAquick Gel Extraction Kit according to the manufacturer's instructions.

2.6.3 Enzymatic Modification of DNA

Restriction of DNA with Restrictionendonucleases

The restriction of DNA was carried out using restriction endonucleases and buffers purchased from NewEngland Biolabs. Conditions and buffers were chosen according to the manufacturer's instructions. The incubation time for the restriction was 2 to 4 h. Finally the digested PCR products or plasmids were purified using the QIAquick PCR Purification Kit according to manufacturer‘s instructions. Digested phage DNA was directly applied to a agarose gel and seperated (2.6.2).

Ligation

For ligation of DNA the T4 DNA ligase (NewEngland Biolabs) according to manufacturer‘s instructions was used. If necessary the 5' phosphate groups were removed to avoid re-circularization of the vector. Therefore 3.5 U of shrimp alkaline phosphatase (NewEngland Biolabs) was added to the vector and incubated at 37 ℃ for 15 min. After 15 min heat activation at 65 ℃ the vector was purified using the QIAquick PCR Purification Kit according to manufacturer‘s instructions. The ligation was carried out using an amount of 100 ng vector DNA mixed with the insert DNA in excess, in which a molar ratio of 1:3 was used. Finally, the ligation was incubated at 20 ℃ for 2-5 h.

2.6.4 Transformation of *Escherichia coli* using $CaCl_2$ competent cells

E. coli DH10B or ST18 were grown aerobically in 100 ml LB at 37 ℃ and 200 rpm. The growth was monitored by measuring the OD_{578}. Starting at an OD_{578} of 0.05 the cells were grown till the culture reached an OD_{578} of 0.6 to 0.7. The culture was chilled on ice for 10 min before harvesting the cells by centrifugation (10 min, 4,000 rpm, Megafuge 1.0 R). The pellet was resuspended in 10 ml of ice cold $CaCl_2$ buffer and again centrifuged as described above. The supernatant was discarded and the cells resuspended in 1 ml of $CaCl_2$ buffer. 50 μl aliquots were prepared, either immediately used for transformation or stored at -80 ℃.
For transformation of *E. coli* by heat shock the competent cells were first thawed on ice for 15 min and than mixed with 10 - 50 ng of plasmid DNA. After incubation on ice for another 20 min the cells were subjected to a heat shock by incubation at 42 ℃ for 45 sec. Afterwards 400 μl fresh LB were added for the recovery of the cells. After incubation at 37 ℃ for 30 to 60 min different volumes were streaked on LB agar plates containing the appropiate antibiotics and plates were incubated overnight at 37 ℃.

$CaCl_2$ buffer:	100	mM $CaCl_2$
	10	% glycerin
		in dH_2O

2.6.5 Preparation of Plasmid DNA

To prepare the plasmid DNA the alkaline lysis method described in Sambrook (1989) was used. 4 ml of an overnight culture was harvested by centrifugation for 2 min at 4 ℃ (13,000 rpm, Heraeus Biofuge fresco). The pellet was resuspended in 300 μl buffer P1. After addition of 300 μl buffer P2 the sample was carefully mixed by inverting and incubated at RT for 5 min. Next 300 μl of buffer P3 were added, again carefully mixed by inverting and incubated for 5 min at RT. After centrifugation for 20 min at 4 ℃ (13,000 rpm, Heraeus Biofuge fresco) 800 μl of the supernatant was mixed with 0.7 volumes of isopropanol and incubated for 10 min at RT. The

precipitated plasmid DNA was centrifuged for 15 min (13.000 rpm, 4 ℃, Heraeus Biofuge fresco) before washing with 70 % ethanol. After all traces of ethanol had evaporated, the DNA was solubilized in 50 μl dH_2O and stored at -20 ℃.

P1:	50	mM Glucose
	25	mM Tris HCl, pH 8.0
	10	mM EDTA, pH 8.0
		in dH_2O
P2:	0.2	N NaOH
	1	% SDS
		in dH_2O
P3:	3	M Na Acetat, pH 5.3
		in dH_2O

2.6.6 Diparental Mating of *Pseudomonas aeruginosa*

The *E. coli* strains ST18 (Thoma & Schobert, 2009) contains the *tra*-genes which are necessary for conjugation. Thereby it is possible to transfer a plasmid from *E. coli* ST18 to *P. aeruginosa* via conjugation. Moreover, the strain ST18 contains a *hemA* deletion resulting in a defective tetrapyrrole biosynthesis. This mutation is of great advantage since counterselection of the *E. coli* donor strain is not required (Thoma & Schobert, 2009).

Overnight cultures of the donor strain *E. coli* ST18 and the recipient *P. aeruginosa* were made. For the diparental mating 1 ml of the *E. coli* ST18 culture was mixed with 100 μl of the *P. aeruginosa* culture and centrifuged for 1 min at 13,000 rpm (Eppendorf MiniSpin). The cells were resuspended in 100 μl LB medium and dropped on an agar plate, dried for 30 min and finally incubated for 6 h at 37 ℃. During this time the mobilizable plasmid is transferred from *E. coli* ST18 to *P. aeruginosa*.

Afterwards the cells were taken off the LB agar plate and were resuspended in 1 ml

LB medium. Dilutions of the cell suspension were streaked on LB agar plates containing the appropiate antibiotics. Plates were incubated overnight at 37 ℃.

2.6.7 Transpsoson Mutagenesis and Arbitrary PCR

Transposon mutagenesis was performed with the mariner transposon as previously described in Kulasekara *et al.* (2005). Cells of *E. coli* S17λ*pir* (de Lorenzo & Timmis, 1994) carrying the mariner transposon on the plasmid pTB20 and of *P. aeruginosa* PAO1 were harvested from agar plates. OD_{578} of 40 for the *E. coli* strain and OD_{578} of 20 for PAO1 were adjusted in LB medium. 25 μl of each strain was mixed and dropped on an agar plate dried for 30 min and finally incubated for 6 h at 37 ℃. During this time the mobilizable plasmid containing the transposon is transferred. After incubation the cells were scraped and resuspended in 1 ml LB. For selection, the cells were incubated with a ten fold excess of the LPS specific phage JG004 for 30 min at 37 ℃. The cells were plated on LB medium containing 200 μg/ml gentamicin and 10 μg/ml chloramphenicol for the inhibition of the *E. coli* S17λ*pir* strain.

Table 7: List of primer for arbitrary PCR. Primers are described in 5' - 3' orientation.

Primer	Sequence	PCR round
P1 (oJG016)	TCTACGTGCAAGCAGATTACGGTGAC	1
P2 (oJG007)	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT	1
P3 (oJG008)	GGCCACGCGTCGACTAGTACNNNNNNNNNNACGCC	1
P4 (oJG005)	GATATCGACCCAAGTACCGCCACCTA	2
P5 (oJG009)	GGCCACGCGTCGACTAGTAC	2

The insertion of the transposon was identified by arbitrary PCR and sequencing as described previously (O'Toole & Kolter, 1998). Arbitrary PCR consists of two PCR rounds. In the first round one primer (P1) binds specifically to the transposon whereas two other primer (P2 and P3) binding randomly in the genome

of *P. aeruginosa*. In the second round the PCR products of the first round were amplified again by using a primer (P4) which also binds to the transposon and primer P5 which binds specifically to the sequences of primer P2 and P3. The sequences of the primers are summerized in Table 7. The PCR mixture is for both PCR rounds alike and the same as described in 2.6.1. For the second PCR 1 μl of the first PCR product was used after purification using the QIAquick PCR Purification Kit according to manufacturer‘s instructions.

The following PCR conditions were used:

1. PCR	95 ℃	5 min
	94 ℃	30 sec
	30 ℃	30 sec
	72 ℃	1 min

Repeating step two to four 6 times

	94 ℃	30 sec
	45 ℃	30 sec
	72 ℃	1 min
	72 ℃	5 min

Repeating step five to seven 30 times

2. PCR	94 ℃	30 sec
	45 ℃	30 sec
	72 ℃	1 sec
	72 ℃	5 min

Repeating step one to three 30 times

2.6.8 Cosmid Library Screen

Cosmid library screening was performed to complement the mutation of the LPS mutants and to identify the mutated gene. The cosmid library is described elsewhere (Schobert & Görisch, 1999). After introducing the cosmid via mating (2.6.6) in the *P. aeruginosa* LPS mutant TM5 the cells were selected with a tenfold excess of the phage SZ01 which binds specifically to the truncated LPS. Bacteria were incubated for 30 min at 37 ℃ with the phage SZ01 and then plated on LB agar plates containing tetracycline. Single clones were investigated for wild type LPS and the cosmid was isolated. Therefore 10 ml overnight culture of the clones were treated as described in 2.6.5. Before investigation of the cosmid it was first introduced in *E. coli* DH10B via transformation (2.6.4) and then again isolated to gain DNA of higher purity (2.6.5). To determine the size of the cosmid insert restriction enzymes cutting upstream and downstream of the insert were used as described in 2.6.3 and analysed via gelelectrophoresis (2.6.2). Finally the genomic region covered by the insert of the cosmid was identified by sequencing (2.8.1) using primers binding to the multiple cloning site of the cosmid.

2.6.9 Phage DNA Preparation

A phage stock (2.5.10) was incubated with 25 U benzonase (Sigma) at 37 ℃ for 30 min to remove all remaining nucleic acids of *P. aeruginosa* cells which were used for the propagation of the phages.

Phages were precipitated using 10 % polyethylenglycol. Therefore, ice cold buffer 1 was added to the phage stock and incubated 1 h on ice. This was followed by 10 min centrifugation at 4 ℃ and 13,000 rpm (Heraeus Biofuge fresco). The supernatant was discarded and the pellet was resuspended in buffer 2. To break open the envelope of the phages and release the phage DNA, SDS with a final concentration of 0.5 % and proteinase K (50 μg/ml) was added and incubated for 1 h at 56 ℃.

After chilling the sample to RT phenol chloroform extraction was performed to remove the proteins from the phage envelope. The extraction was done at least 3 times until there was no interphase visible anymore.

Precipitation of DNA occured with 2.5 volumes of ice cold 100 % ethanol at -20 °C. After centrifugation for 15 min at 4 °C and 13,000 rpm (Heraeus Biofuge fresco) the DNA was washed twice with 70 % ethanol, solubilized in 50 μl dH_2O and stored at -20 °C.

To identify a linear phage chromosome the isolated DNA was treated with the exonuclease *Bal31* according to manufacturer's instructions. This enzyme degrades only double-stranded linear DNA from both ends simultaneously. The digestion was analyzed by agarose gelelectrophorese (2.6.2).

Buffer 1:	30 % Polyethylenglycl (PEG 6000) 3 mM NaCl in dH_2O
Buffer 2:	10 mM Tris HCl, pH 7.8 5 mM EDTA in dH_2O
SDS stocksolution:	20 % (w/v) SDS in dH_2O
Proteinase K stocksolution:	20 mg/ml in dH_2O

2.7 Construction of *P. aeruginosa* Knockout Mutants

To obtain unmarked gene deletion mutants, the well-established strategies based on *sacB* counter selection and FLP recombinase excision (Hoang *et al.*, 1998) was used. To construct the suicide vectors pJG002 and pJG003 the *BamHI* digested gentamicin resistance cassette of pPS858 was cloned between two PCR fragments in the multiple cloning site of pEX18Ap. The two PCR fragments contained DNA homologs to the upstream and downstream regions of the knockout gene. The primers used to amplify these regions, the size of the PCR product and the restriction

sites are depicted in Table 8 and Table 9.

Table 8: List of primer for *mexR* gene deletion. Primers are described in 5' - 3' orientation. The restriction site is underlined. Primer oJG031 and oJG032 are forward and reverse primer for upstream region. oJG033 and oJG034 are forward and reverse primer for downstream region.

Primer	Sequence	Restriction site	Size
oJG031	CGAGCTCTGAGGATGATGCCGTTCAC	*Sac*I	516 bp
oJG032	CGCGGATCCTGGTTTGGCCGAGTAAA	*Bam*HI	
oJG033	CGCGGATCCTTACTGGCGAATGCCTT	*Bam*HI	450 bp
oJG034	CCCAAGCTTCTGTACGGCCGCTTCAA	*Hind*III	

Table 9: List of primer for PA5001 gene deletion. Primers are described in 5' - 3' orientation. The restriction site is underlined. Primer oJG099 and oJG100 are forward and reverse primer for upstream region. oJG101 and oJG102 are forward and reverse primer for downstream region.

Primer	Sequence	Restriction site	Size
oJG099	CGAGCTCCCGCAGGCATTGTTGCACTA	*Sac*I	402 bp
oJG0100	CGCGGATCCCAGCATGGCGGCTCGAA	*Bam*HI	
oJG101	CGCGGATCCAGTAGCAGCCGAAAAGA	*Bam*HI	516 bp
oJG102	CCCAAGCTTCATGTGTGCCACCTGTT	*Hind*III	

The knockout constructs were first introduced into *E. coli* DH10B by transformation (2.6.4). Plasmids of carbenicillin and gentamicin resistant cells were isolated (2.6.5) and controlled by restriction analysis (2.6.3).

Finally, the plasmids were transferred to *P. aeruginosa* via a diparental mating (2.6.6). For selection, the cells were plated first on LB agar plates containing gentamicin. The pEX18Ap plasmid is not able to replicate in *P. aeruginosa*. Cells

can only grow when the gentamicin resistance casette was integrated into the genome. This integration occurs upstream and downstream of the knockout gene due to the homologue regions. A single cross over leads to integration of the plasmid but not to a deletion of the gene itself. Therefore a double cross over is required. Clones were selected on LB agar plates containing 5 % sucrose and gentamicin. The plasmid pEX18Ap contains a *sacB* gene which encodes a levansucrase. A toxic product is produced by this protein in the presence of sucrose. Due to this selection only carbenicillin sensitive clones with integrated gentamicin casette and removed plasmid can grow. The clones were stored at -80 ℃ (2.5.5).

2.7.1 Verification of *Pseudomonas aeruginosa* Knockout via PCR

Removal of Gentamicin Resistance Cassette

After a successful knockout the gentamicin resistance cassette was removed from the genome. This resistance cassette from pPS858 is flanked by *FRT* (Flp recombinase target site) recognition sequences. By using a Flp-recombinase the resistance cassette could be removed from the genome (Hoang *et al.*, 1998). The Flp-recombinase which is encoded on the plasmid pFLP2 binds to the *FRT* recognition sequence and removes the interjacent parts from the genome. The plasmid pFLP2 was first introduced into *E. coli* ST18 by transformation (2.6.4) and then transferred into *P. aeruginosa* via a diparental mating (2.6.6). For selection, the cells were plated first on carbenicillin. During the expression of the plasmid in *P. aeruginosa* the Flp-recombinase catalyzes the excision of the resistance cassette and leaves a single FRT sequence of approximately 150 bp. After excision the cells were plated on LB with 5 % (w/v) sucrose to remove the plasmid pFLP2. This plasmid contains the *sacB* gene, encoding a protein which produces a toxic product when sucrose is present. Therefore only cells which lost the plasmid can grow on sucrose. Finally the clones were tested on carbenicillin and gentamicin to test if the gentamicin resistance cassette was removed and the plasmid pFLP2 is eliminated.

PCR Verification

To verifiy the knockout mutant a PCR was performed (2.6.1). The primer which bind upstream and downstream of the homologue sequences were used and are listed in Table 8 and Table 9. During PCR the DNA upstream and downstream of the excised gene was amplified. As a control the same PCR was performed with the wild type PAO1. The PCR products were documented with agarose gelelectrophorese (2.6.2) and compared with a DNA size standard.

2.8 DNA Sequencing

2.8.1 Sequencing of Single Genes

DNA sequences were obtained with an Abi Prism^{TM} 310 Genetic Analyzer in our institute. The required preparatory PCR with fluorescence-labeled ddNTPs and purification of the PCR product were carried out as described by the manufacturer. The primer used to amplifiy single genes are listed in Table 10.

Table 10: List of primer used for amplification of single genes. Primers are described in 5' - 3' orientation.

Primer	Sequence	Amplified gene	Size
oJG019	AAGACTTCGGCATCAAGATGGACCTC	*mexR*	735 bp
oJG020	TTTTAGCTCGATGGCCGGTTATCCAC		
oJG021	AAATGACCAGCGTCAACCCTAACGAG	*nalC*	836 bp
oJG022	GTCACCGAGATCCA CCTCACCGAACT		
oJG023	TCGAAATACTTCGAGTCCGCCCGAGC	*nalD*	772 bp
oJG024	CCGGCTCGACAGCACCCAGGTACT		
oJG095	TGCGTCGGCACGAGTTGTTC	PA5001	1177 bp
oJG096	TGCGATCTTCATCGGTCCTA		

2.8.2 Sequencing of Phage Genomes

Whole genome sequencing of the phages JG004 and JG024 was done at the McGill Universtiy and Génome Québec Innovation Centre (Montréal, QC, Canada) using 454 Technology. A 71 fold coverage of the genomes was achieved.

2.8.3 Annotation of Phage Genomes

The annotation of the unknown phage genes were done by using the software GeneMark.HMM (Besemer & Borodovsky, 1999) (http://exon.biology.gatech.edu/). The Heuristic approach of GeneMark was used to identify genes in small genomes under 100 kb. The identified genes were compared with the NCBI ORF finder (Wheeler *et al.*, 2003) (http://www.ncbi.nlm. nih.gov/gorf/gorf.html). Nucleotide sequences were scanned for homologues using the Basic Alignment Search Tool (blastx) (Altschul *et al.*, 1997) (http://blast.ncbi. nlm.nih.gov/Blast.cgi). To search for tRNA genes in the phage sequences the internet tool tRNAscan-SE 1.21 (Lowe & Eddy, 1997) (http://lowelab.ucsc.edu/tRNA scan-SE/) was used.
Sequence comparison and distance phylograms with the PB1-like phages was conducted using ClustalW2 online analysis tool (Larkin *et al.*, 2007) (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Investigation of the codon usage was performed using a software tool based on JCat (Grote *et al.*, 2005).

2.9 Preparation and Visualization of LPS

To investigate the LPS from *P. aeruginosa* the LPS was isolated and analyzed by a two-buffer tricine-based SDS-PAGE system. The isolation of the LPS was performed as described previous by Coyne *et al.* (1994). First a bacterial lawn on LB agar plates was prepared and incubated over night at 37 °C. The cells were harvested and resuspended in 1 ml PBS buffer to an OD_{578} of 10. The cell suspension was autoclaved and mixed for at least 1 min. After centrifugation for 2 min and 13,000 rpm (Eppendorf MiniSpin) the supernatant was mixed with a fourfold volume of 95 % ethanol. This was followed by a precipitation at -20 °C over night and a centrifugation for 10 min at 13,000 rpm (Eppendorf MiniSpin). The pellet was

resuspended in 200 μl of PBS buffer. Benzonase was added with a concentration of 3 U and incubated for 2 h at 37 ℃. In the end Proteinase K (100 μg/ml) was added and the sample was incubated at 56 ℃ over night to remove all the remaining proteins. The isolated LPS was stored at -20 ℃ until analysis. The two-buffer tricine-based SDS-PAGE is based on Schägger & von Jagow (1987) and consists of a 4 % stacking gel and a 16.5 % separating gel. Before analysis by SDS-PAGE, 3 μl of the LPS sample was combined with an equal volume of 2 x sample buffer and heated to 95 ℃ for 5 min. For seperation of the LPS a cathode buffer with a pH of 8.25 and an anode buffer with a pH of 8.9 was used. First an amperage of 15 mA was applied until the sample reached the seperating gel then the amperage was elevated to 30 mA. Before silver staining with 0.1 % silver nitrate the gels were incubated in acetic acid for 30 min. After 5 min washing in dH_20 the gels were developed in 2.5 % sodium carbonate solution for 2-5 min. To stop the development, the gels were transferred into a stop solution.

Acrylamide stock solution	Rotiphorese Gel 30 (37.5:1) (Roth)	
Anode buffer	200	mM Tris
		in dH_2O, pH 8.9
Cathode buffer	100	mM Tris
	100	mM Tricine
	0.1	% (w/v) SDS
		in dH_2O, pH 8.25
Gel buffer	3.0	M Tris
	0.3	% (w/v) SDS
		in dH_2O, pH 8.45

SDS sample buffer	0.2	% Bromphenolblue
	10	% β Mercaptoethanol
	40	% Glycerol
	3.2	% (w/v))SDS
	100	mM Tris HCl
		in dH_2O, pH 6.8
4 % stacking gel	670	μl Acrylamide stock solution
	630	μl Gel buffer
	1.2	ml dH_2O
	2.5	μl Tetramethylen diamine (TEMED)
	17.5	μl APS (10 % (w/v) in dH_2O)
16.5 % seperating gel	2.75	ml Acrylamide stock solution
	1	ml Gel buffer
	250	μl Glycerol
	5	μl Tetramethylen diamine (TEMED)
	50	μl APS (10 % (w/v) in dH_2O)
Fixing solution	10	% (v/v) Acetic acid
Staining solution	0.1	% (w/v) Silver nitrate
		in dH_2O
Developer	2.5	% (w/v) Sodium carbonate
	0.1	% (v/v) Formaldehyde
	0.001	% (w/v) Sodiumthiosulfate
		in dH_2O

Stop bath	2	% (w/v) Glycine
	0.5	% (w/v) EDTA
		in dH_2O

2.10 Preparation and Visualization of Outer Membrane Proteins

To investigate outer membrane proteins of *P. aeruginosa* the proteins were isolated as previously described in Carlsohn *et al.* (2006). Twenty ml of a *P. aeruignosa* overnight culture were pelleted and washed twice with 20 mM Tris-HCl, pH 7.5. Three units of benzonase (Sigma) and 0.1 mg/ml protease inhibitor were added. The cells were broken by repeated ultrasonication (5 x 2 min). Incubation for 30 min at room temperature to digest the nucleic acid was followed by 10 min centrifugation at 4000 rpm (Heraeus Megafuge) to remove cell debris. The supernatant was centrifuged at 50.000 x g for 40 min. The pellet was dissolved in 5 ml 20 mM Tris-HCl, pH 7.5 and 2 % (w/v) sodium lauryl sulfate. Incubation for 30 min at room temperature led to precipitation of the proteins. A final centrifugation for 30 min at 50.000 x g precipitates the proteins which can be dissolved in 20 μl XT sample buffer. The protein concentration was determined using the 2D-Quant Kit (Amersham Biosciences) according to manufacturer‘s instructions. Fifty μg protein were loaded on a 15 % SDS gel for electrophoretic seperation of the outer membrane proteins. For visualization of the proteins the gels were stained with ruthenium(II)-tris-(bathophenanthroline disulfonate) as described in Rabilloud *et al.* (2001). Protein identification was performed using mass spectrometry as described in Schreiber *et al.* (2006).

XT sample buffer	62.5	mM Tris-HCl
	25	% Glycerol
	2	% SDS
	0.01	% Brominephenolblue
	5	% Mercatoethanol
		in dH_2O

3 Results and Discussion

3.1 Characterization of Two *P. aeruginosa* Broad Host Range Phages

During this work 58 *P. aeruginosa* specific phages were isolated from the sewage treatment plant Steinhof in Braunschweig. The aim was to isolate and characterize lytic broad host range phages which infect different *P. aeruginosa* strains. Two phages, JG004 and JG024, were selected for detailed characterization.

3.1.1 Host Range of Phage JG004 and JG024

The term "host range" of a phage is defined by which bacterial genera, species and strain a phage can lyse. *P. aeruginosa* shows clonal differences since it depicts a core genome plus variable accessory segments (Wiehlmann *et al.*, 2007). These variable segments are mobile islands and elements which can be exchanged between strains and therefore cause a striking diversity among the *P. aeruginosa* population (Pirnay *et al.*, 2009). These diversity can also afflict the receptors of the phages like the LPS. There are 20 major serogroups of *P. aeruginosa* known reflecting only the diversity of the O-antigen (King *et al.*, 2009). To investigate the host range of the phages we used different isolates of CF patients, urinary tract infections as well as a set of environmental strains to cover as much as possible different *P. aeruginosa* clones. Table 11 shows the used clinical *P. aeruginosa* isolates and wild types as well as the results of the host range for phage JG004 and JG024. JG004 is able to infect nearly 48 % of the tested strains, whereas JG024 is able to infect even 85 % of all tested strains. The phage JG004 showed a clear lysis in 50 % of the infection events. For JG024 a clear lysis was observed for 83 % (Tab. 11). Also, both phages were able to infect a *mucA* mutant strain. This mutant produces large amounts of exopolysaccharides which results in a mucoid phenotype (Martin *et al.*, 1993) suggesting that mucoidy does not protect clinical isolates from phage infection.
Moreover, in cooperation with Marlon Kazmierczak the host range of the isolated phages described in this work was determined with a collection of 100 environmental

Table 11: Host range of phage JG004 and JG024 with clinical isolates. Lysis of bacteria was determined as described in 2.5.13 and catergorized as clear (+), turbid (0) or no lysis (-). Clear lysis indicates a complete lysis, whereas a turbid plaque indicates partiell lysis.

P. aeruginosa strain	JG004	JG024
PAO1	+	+
PA14	-	+
BT2	-	+
BT72	-	0
BT73	-	+
RN3	-	+
RN43	-	+
RN45	0	+
NN84	-	-
PACF15	-	+
PACF21	+	+
PAKL1	-	-
PAKL4	0	0
PACF60	+	+
PACF61	+	+
PACF62	-	+
PACF 63	0	+
$\Delta mucA$	+	+
MH 18	0	-
MH 19	+	+
MH 26	0	0
MH 29	-	+

P. aeruginosa strains. The strains were isolated from different rivers (Oker, Aller, Weser) in Lower Saxony, Germany. JG004 was able to infect 49 % of the tested strains, whereas JG024 was able to infect 50 % of the strains (Fig. 4). Interestingly, JG004 showed a clear lysis for 90 % of the tested strains whereas JG024 showed a clear lysis only in 45 % of the cases.

These still limited number of infection experiments indicate that JG024 is able to infect the clinical isolates more effective than JG004 whereas JG004 infect environmental isolates more efficiently. However, the presented results indicate that both phages, JG004 and JG024, have a broad host range activity.

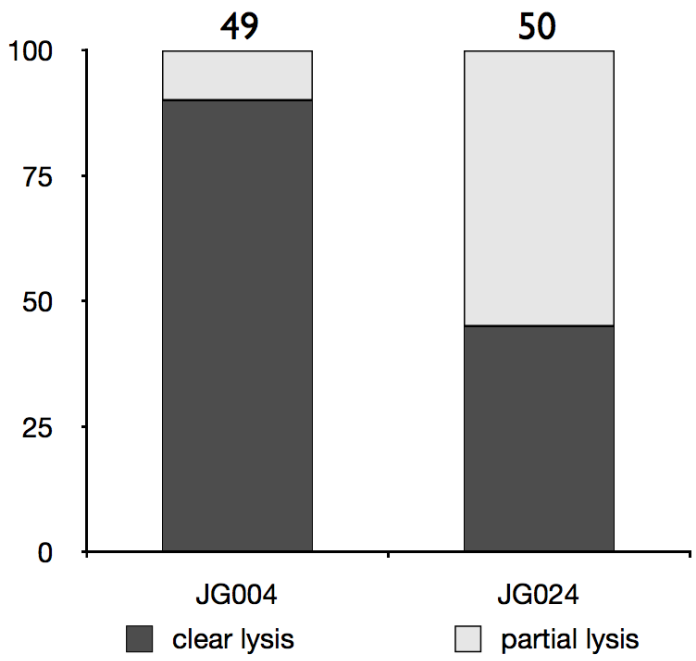


Figure 4: Host range of JG004 and JG024. One hundred environmental *Pseudomonas aeruginosa* strains were tested. JG004 was able to infect 49 strains, JG024 infected 50 strains. The dark grey area indicated the clear lysis in percent. Light grey indicated a partial lysis in percent.

In comparison to the described results, Knezevic *et al.* (2009) isolated 19 *P. aeru-*

ginosa phages with broad host spectrum and identified phages which infected between 15-76 % of 33 tested *P. aeruginosa* strains. The used *P. aeruginosa* strains of this study were isolated from human and animal pathological material (22 strains) as well as from the environment (10 strains). Moreover, five strains belonging to the familiy *Enterobacteriaceae* were also used for the host range experiments but none of the phages was able to lyse these species.

3.1.2 Receptor of Phage JG004 and JG024

Diverse structures on the surface of Gram negative bacteria, as the LPS, outer membrane proteins, flagella and pili can serve as receptors for phages (Kutter & Sulakvelidze, 2005). To identify the receptor of the phages JG004 and JG024, different *P. aeruginosa* mutant strains which lack flagella (Δ*fliM*), pili (Δ*pilA*) or a complete LPS (Δ*algC*) were used.

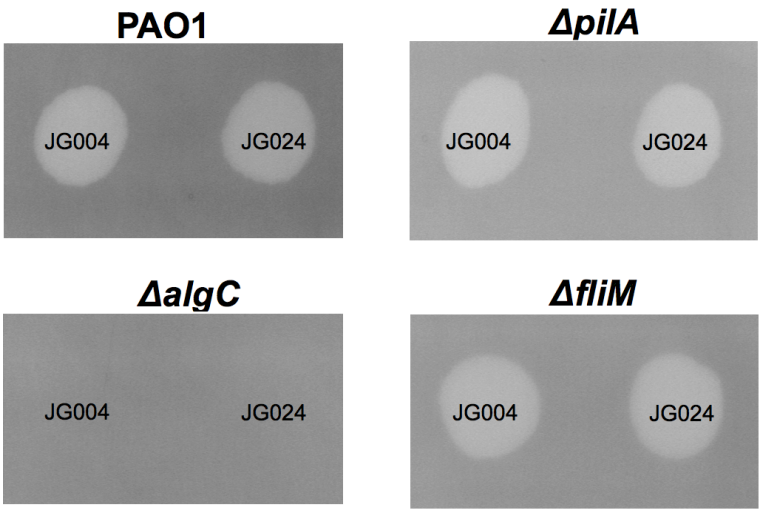


Figure 5: Identification of potential receptors of phage JG004 and phage JG024. Phages JG004 and JG024 were directly spotted onto bacterial lawns containing the indicated *Pseudomonas aeruginosa* wild type and mutant strains. *P. aeruginosa* mutant strains lack flagella (Δ*fliM*), pili (Δ*pilA*) or a complete LPS (Δ*algC*). Clear zone indicates infection of the strain.

The gene *algC* is needed for a complete core in *P. aeruginosa* LPS (Coyne *et al.*, 1994). Phages were directly spotted onto an bacterial lawn as described in 2.5.13. After incubation, lysis was determined. Both phages were able to lyse the *P. aeruginosa* flagella and pili mutant but not the LPS mutant (Fig. 5). Since both phages are not able to infect this mutant it is likely that the phages specifically bind to the LPS core.

3.1.3 Family Characteristics - Morphology and Nucleic Acid

As described in 1.1 there is no numerical taxonomy for viruses and classification is based in the morphology and the nature of the nucleic acid.

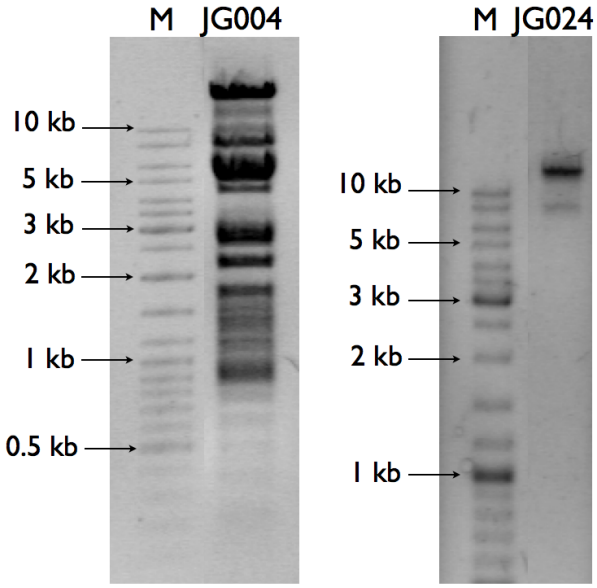


Figure 6: Restriction pattern of dsDNA of phage JG004 and JG024. Shown are images of 1 % agarose gels stained with ethidiumbromide. DNA of JG004 was digested with *Hind*III and DNA of JG024 with *Sac*II. M: Molecular weight marker.

The vast majority of phages contain a single DNA molecule (Abedon, 2009). Therefore, the DNA of the phages was isolated as described in 2.6.9. After the isolation,

the DNA was digested with restriction endonucleases (2.6.3) which cleave exclusively dsDNA. The DNA was successfully digested indicating that both phages contain ds-DNA (Fig. 6). The DNA of phage JG004 was digested by *Hind*III whereas the DNA of JG024 was digested by *Sac*II. *Hind*III couldn't cleave the DNA of JG024 suggesting that there are no *Hind*III recognition sites present in the respective phage genome or the phage DNA contains modified bases preventing digestion by *Hind*III.

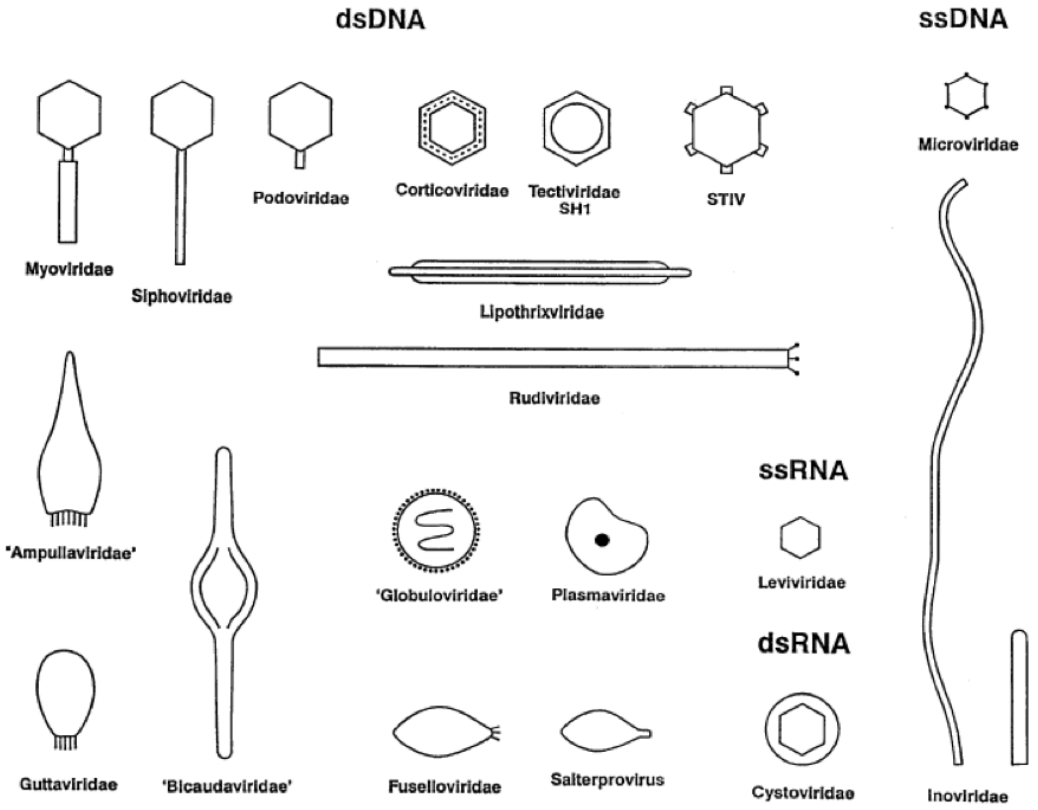


Figure 7: Known morphotypes of bacteriophages (Ackermann, 2007)

Moreover, different restriction patterns indicate different phages. In this case it is obvious that the DNAs of the phages are different since it was not possible to cleave the DNA of JG024 with *Hind*III in comparison with JG004 (Fig. 6). Also, the

DNA of JG024 could not be cleaved by the restriction endonuclease *Ssp*I whereas the DNA of JG004 was cleaved by *Ssp*I (data not shown) which underlines the difference between these phages.

Fig. 7 shows the known morphotypes of phages. Each phage family has a characteristic morphology, therefore, phages can be easily assigned to a family by comparison. To investigate the morphology of the phages JG004 and JG024 electron microscopical pictures were taken (Fig. 8).

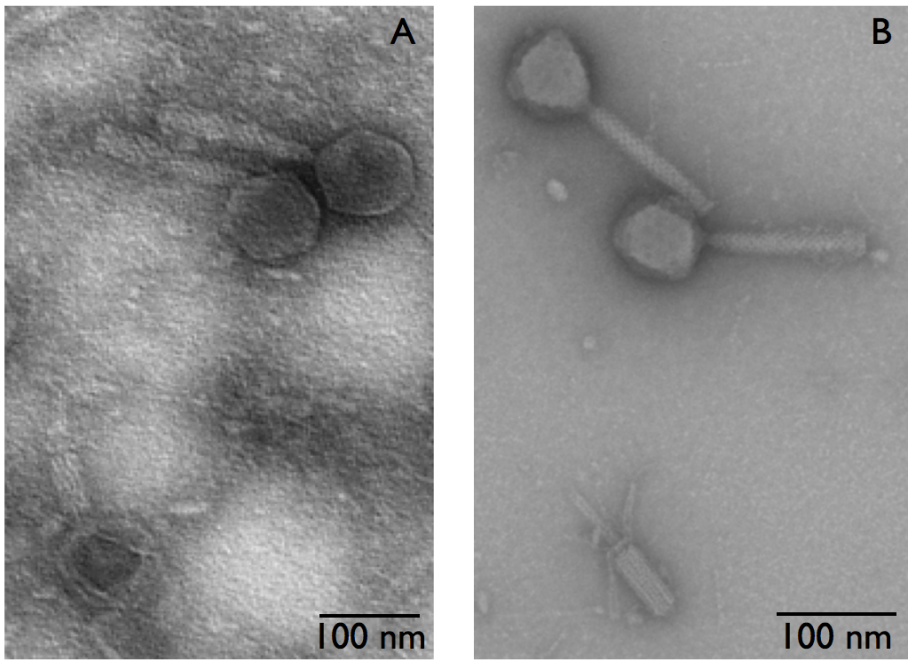


Figure 8: Electron microscopical picture of phage JG004 (A) and JG024 (B). Pictures were taken by M. Rohde at the HZI to allow family affiliation which is based on the morphology of the phages. Both phages have a contractile tail, JG004 with a length of 150 nm and JG024 with a length of 130 nm. The head of JG004 has a length and a width of 60 nm whereas the head of JG024 has a length of 80 nm and a width of 75 nm. Therefore both phages belong to the family *Myoviridae*

Both electron microscopical pictures show tailed phages. The tail of the phages are contractile and consist of a neck, a contractile sheath and a central tube. It

can be concluded that these phages belong to the family *Myoviridae* due to their morphology and their dsDNA. The family *Myoviridae* belongs with two other tailed phage families, *Siphoviridae* and *Podoviridae*, to the order *Caudovirales*. Tailed phages are probably extremely ancient because they infect both Eubacteria and Archaea. Moreover, they are the most numerous and ubiquitous of all viruses. 25 % of tailed phages belong to the family *Myoviridae* (Clokie & Kropinski, 2009).

3.1.4 Growth Characteristics

A number of parameters like the latent phase and the burst size are known to describe the infection cycle of a phage.

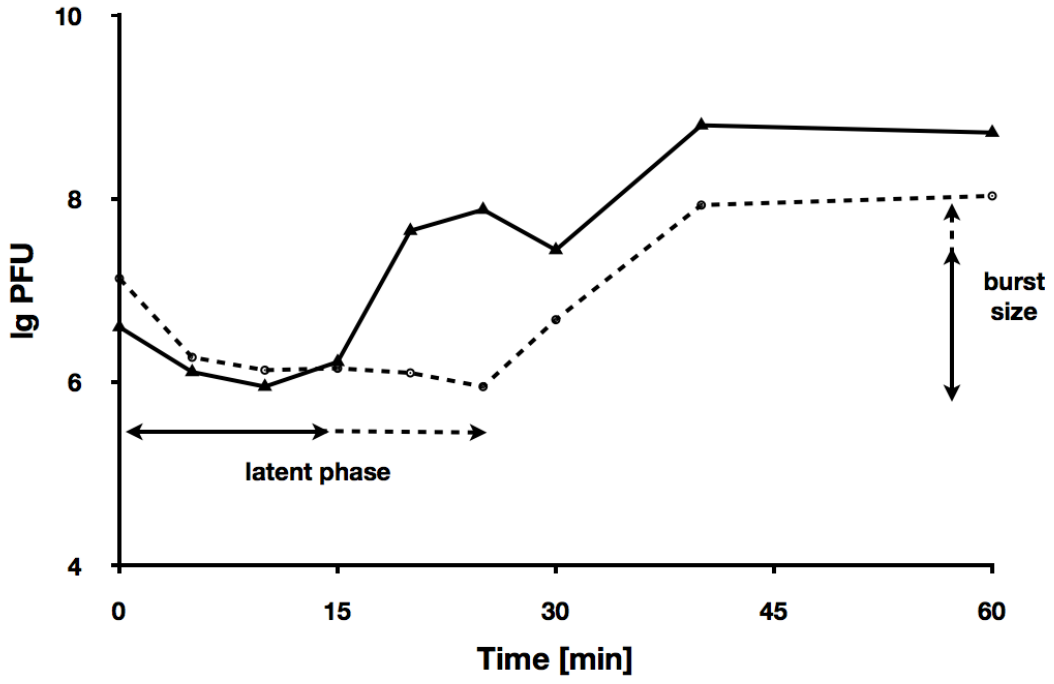


Figure 9: One step growth curve of phage JG004 (solid line) and phage JG024 (dashed line). Cells from exponential growth phase were infected with phages in a 3:1 ratio and incubated at 37 °C as described in 2.5.15. Samples to determine the phage titer were taken at indicated time points. Arrows indicate the latent phase and the burst size of the phages.

To determine these parameters one step growth curves were performed (2.5.15). Thereby, the bacteria and viruses in this experiment were mixed in a ratio that only one phage is likely to adsorb to a bacterium. Moreover, it is best to infect bacteria in a exponential growth phase to have metabolic active cells (Kutter & Sulakvelidze, 2005). Fig. 9 shows the single step growth curves of the phages JG004 and JG024 by comparison. Phage infection starts with the attachment to the host bacterium and the injection of the nucleic acid into the host. The number of free phages decreases during this phase which can be observed in the single step growth curve. The time from infection until the first new, infective phages are released from the cell is called latent phase as indicated in Fig. 9. The latent phase for phage JG004 is shorter than for phage JG024. The latent phases could be estimated as 15 min for JG004 and 25 min for JG024 (Tab. 12).

After the latent phase, the number of phages in the culture increases suddenly which represents the number of released phage progeny. The burst size which describes the mean number of phages liberated per bacterial cell was calculated as described in Material and Methods (2.5.15). Wang (2006) pointed out that the duration of the latent phase correlates with the burst size. The longer the latent phage the bigger the burst size of the phage. Since phage JG024 has a longer latent phase this phage is able to produce approximately 120 phages per infected cell. On the other hand, phage JG004 has a short latent phase of only 15 min and the burst size is therefore smaller with only 27 phages per infected bacterium (Tab. 12).

Table 12: Growth characteristics of phage JG004 and JG024

Phage	Duration of infection	Burst size
JG004	15	27 phages per cell
JG024	25	120 phages per cell

Moreover, the phage JG004 is able to carry out two infection cycles in the same time JG024 is able to carry out only one infection cylce (Fig. 9). The growth curve

of JG004 describes a first peak after a first burst at around 25 min. Afterwards, the number of phages decreases again due to binding of the phages to bacteria and finally a second burst increases the total number of phages.

3.1.5 Sequence Analysis of JG004 and JG024

Phage genomes were sequenced using the 454 Technology at the McGill Universtiy and Génome Québec Innovation Centre (Montréal, QC, Canada).
General properties like the genome size, the GC content and the number of possible ORFs are summarized in Table 13. The GC content and the annotation of genes were done by using the software GeneMark (Besemer & Borodovsky, 1999). The possibility of genes coding for tRNAs was investigated using the tool tRNAscan-SE 1.21 (Lowe & Eddy, 1997).

Table 13: General properties of the JG004 and JG024 genome.

Phage	Genome size	GC content (%)	predicted ORFs	tRNA genes
JG004	93,010 bp	49.26	160	12
JG024	66,275 bp	55.62	94	no

The genome size of phage JG024 is 66,275 bp, with 94 putative ORFs and has a GC content of 55.62 %. No genes encoding for tRNAs were found. The genome of phage JG004 is significantly larger with 93,010 bp, 160 putative ORFs and a GC content of 49.26 %. Moreover, the genome of phage JG004 contains 12 tRNA genes (Tab. 14), the cloverleaf structure is shown in Fig. 29 (Appendix).
Normally, phages utilize the host's protein synthesis machinery to synthesize their own proteins and enzymes, but some phages encode their own tRNAs. tRNAs were most commonly found in the members of the *Myoviridae* with large genomes (Clokie & Kropinski, 2009). Kropinski & Sibbald (1999) pointet out that tRNA genes in phages are almost always clustered and that they may facilitate a more rapid overall

Table 14: tRNAs of phage JG004.

tRNA	Begin	End	Amino Acid	Anti Codon	Length
1	58738	58666	Glu	TTC	72
2	58821	58748	Phe	GAA	73
3	58903	58831	Gly	TCC	72
4	58987	58913	Pro	TGG	74
5	59126	59054	Asn	GTT	72
6	59212	59140	Cys	GCA	72
7	59700	59625	Asp	GTC	75
8	59785	59713	Ile	GAT	72
9	60080	59999	Leu	TAG	81
10	60431	60358	Lys	TTT	73
11	60513	60442	Arg	TCT	71
12	60892	60822	Gln	TTG	70

translation rate, especially the translation rate for rare codons. Interestingly, the tRNA genes in JG004 are also clustered and since this phage has a really short infection cycle of less than 15 min the phage tRNAs could be helpful to achieve a higher overall translation rate. They may function in the translation of certain highly produced proteins since the GC content of phage JG004 with 49.26 % differs from the GC content of the host *P. aeruginosa* with 68 %. Comparison of the codon usage with the host showed that phage JG004 shares the same dominant codons for the following amino acids: isoleucine (AUC), tyrosine (UAC), cysteine (UGC), histidine (CAC), tryptophan (UGG), asparagine (AAC), lysine (AAG), leucine (CUG), aspartic acid (GAC), phenylalanine (UUC) and glutamic acid (GAG) (Tab. 18, Appendix). Interestingly, phage JG004 features own tRNAs for the dominant codons of phenylalanine (UUC), aspartic acid (GAC), asparagine (AAC), isoleucine (AUC) and cysteine (UGC) probably to achieve a higher overall translation rates. Moreover, the other tRNAs ensure a translational efficiancy for codons which are not so

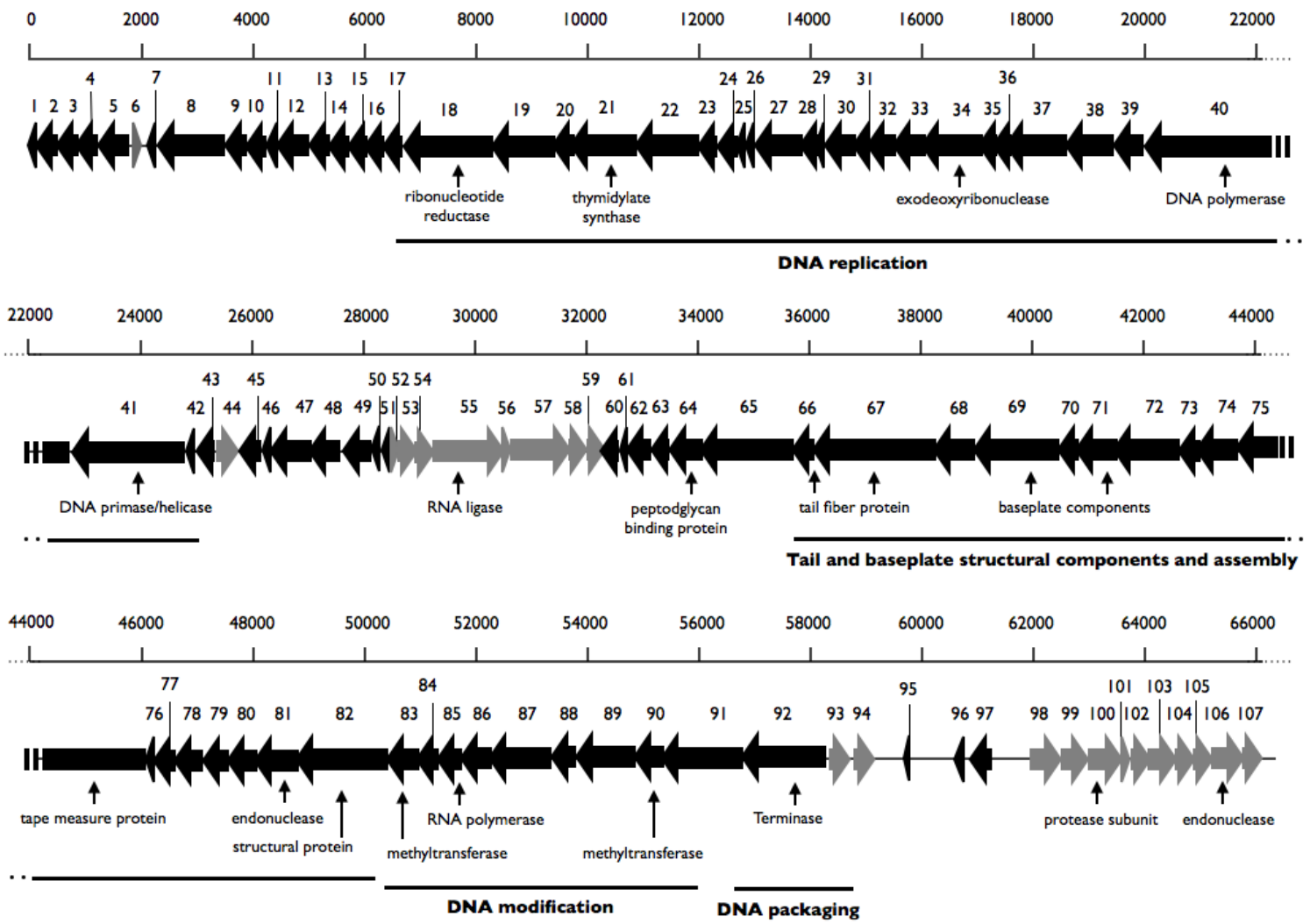
freuquently or rarely used in *P. aeruginosa* as in the phage JG004 (glutamic acid (GAA), glycin (GGA), proline (CCA), leucine (CUA), lysine (AAA), arginin (AGA) and glutamine (CAA)) (Tab. 18, Appendix).
Interestingly, the GC content of phage JG024 also differs from its host (55.62 % to 68%). Comparison of the codon usage of JG024 with its host *P. aeruginosa* showed that the phage shares the same dominant codons for each amino acid exept for valin, serin and glutamat and therefore ensuring translational efficiancy (Tab. 18, Appendix).

The annotated genes and their predicted function are listed in Table 19 and Table 20 of the Appendix. The schematic representation of the genomes, with their predicted ORFs, some functional assignments and overall genetic organization are depicted in Fig. 10 and 12.

Features of JG004 Genome

Eighty three (52 %) of the 160 predicted ORFs show no similarities to other genomes in databases. The genome of phage JG004 shows 11.3 % intergenic space. This is comparable with the host *P. aeruginosa* PAO1 which shows 10.6 % non coding regions (Stover *et al.*, 2000). Some bigger non coding regions of the JG004 genome were observed in the area of the coding regions for the tRNAs and between ORF 141 to 145.

Since ORF 85 has similarities to RNA polymerases, the phage JG004 is probably not dependant on the host transcriptional machinery. Moreover, genes encoding for the DNA replication machinery were found, suggesting that the DNA replication is also independent from the host. We found ORFs with similarities to a DNA polymerase (ORF 40), a DNA helicase/primase (ORF 41), a thymidylate synthetase (ORF 21), a ribonucleotide reductase (ORF 18) and for a exodeoxyribunuclease (ORF 34). A terminase like gene (ORF 92) could also be detected. Phage terminases are the DNA packaging enzymes and among the most conserved proteins found in phages.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16000
18000
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22000
ribonucleotide reductase
thymidylate synthase
exodeoxyribonuclease
DNA polymerase
DNA replication
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36000
38000
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42000
44000
DNA primase/helicase
RNA ligase
peptodglycan binding protein
tail fiber protein
baseplate components
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RNA polymerase
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endonuclease
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DNA packaging

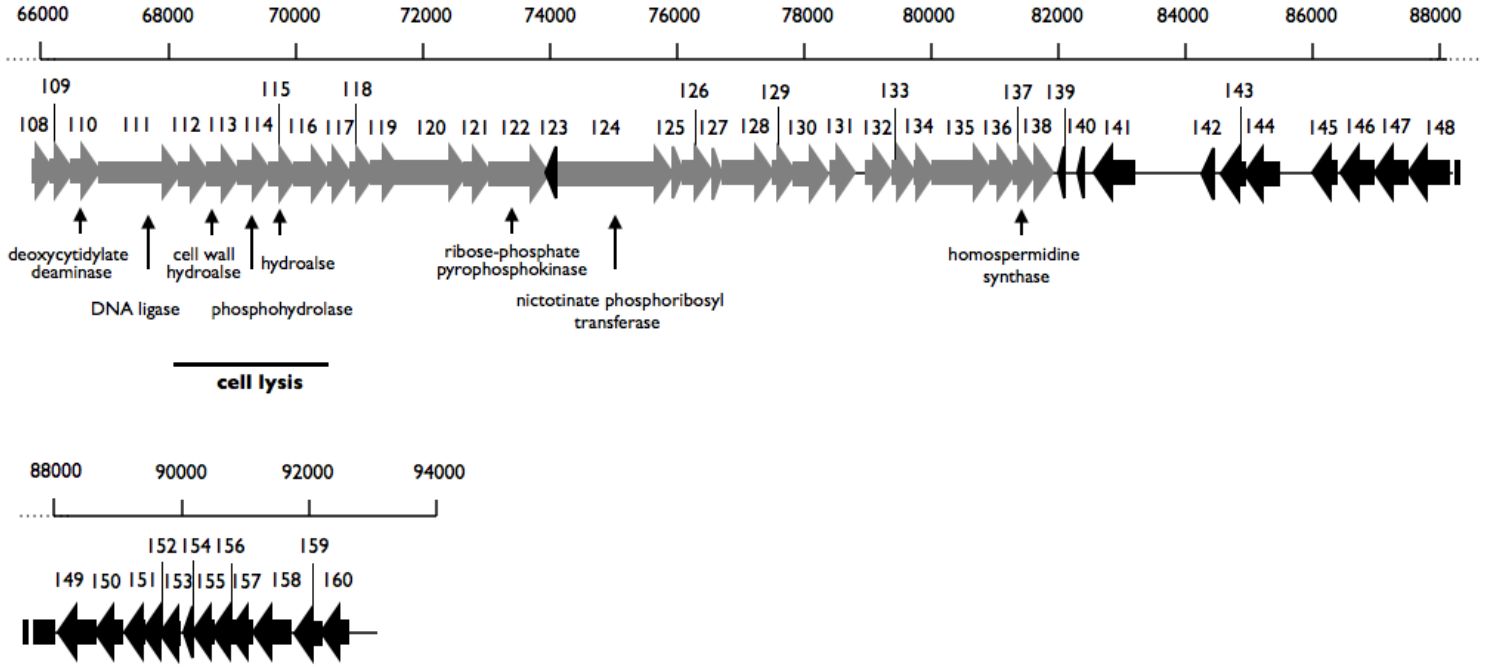

Figure 10: Schematic representation of the JG004 genome with its assumed ORFs and some functional assingments. The arrowheads point in the direction of transcription.

Terminases have ATPase activity that powers the DNA translocation into the virion. Some terminases also contain endonuclease activity to cut the DNA into genome length (Rao & Feiss, 2008).
Two putative endonucleases were also detected (ORF 81, 106). These endonucleases could be involved in the DNA packaging process or in host nucleic acid damaging. Moreover, two putative methyltransferases were found. Methyltransferases are important for the methylation of DNA to protect the DNA against own endonucleases or endonucleases of the host which serve as a protection against foreign DNA and infection of phages.

Only five ORFs were found with similarities to phage particle proteins. Two of them are putative tail fiber proteins (ORF 66 and 67) and two of them are putative baseplate components (ORF 69 and 71). ORF 82 has similarities to a putative structural protein of the *Erwinia* phage phiEa21-4. None of these proteins have similarities to head structural proteins, suggesting that the phage is not related to any other sequenced phage since it contains unique structural proteins.
A putative tape measure protein was also detected (ORF 75) close to the putative structural proteins. It was shown for phage T4 that the so called tape measure protein regulates the length of the phage tail (Abuladze *et al.*, 1994). Moreover, it was described for Mycobacteria specific phages that the tape measure protein facilitates phage entry into stationary phase cells with thickened peptidoglycan layer due to a peptidoglycan hydrolase motif (MT3 motif) associated with the tape measure protein (Dusthackeer *et al.*, 2008).

Lysis of bacteria by phages with dsDNA is accomplished by two main proteins, an endolysin which degrades the peptidoglycan and a holin which permeabilize the cytoplasmic membrane to facilitate entry of the endolysin into the periplasm (Young *et al.*, 2000). We found one putative cell wall hydrolase (ORF 113) among the identified ORFs. This hydrolase has similarities to the cell wall hydrolase SleB of *Methylobacterium populi* but not to endolysins of other phages. A conserved protein domain of the hydrolase 2 superfamily was also detected in this putative cell wall

hydroalse. However, we couldn't detect any similarities to a holin which is likely because holins are very diverse and a are classified into twelve unrelated orthologous groups (Young *et al.*, 2000).

Relatively small proteins with less than 100 amino acids were found in in the genome of phage JG004, the smallest one with only 34 amino acids. None of these small proteins has a predicted function. It was shown before that phage genomes contain small proteins with unknown function (Miller *et al.*, 2003; Ceyssens *et al.*, 2006; Weigele *et al.*, 2007). It is speculated that these proteins may have a role as accessory factors that bind to and subtly modify the specificity of host proteins so that they function appropriately during phage infection (Mann *et al.*, 2005).

Finally, a putative homospermidine synthase was found (ORF 137), although with sparse identity (34 %, e-value: 0.32). A homospermidine synthase produces homospermidine and propane-1,3-diamine out of spermidine and putrescine. Homospermidine is a aliphatic polyamine (Tholl *et al.*, 1996). Although it was found in some bacteria (Hamana & Matsuzaki, 1992), plants (Ober & Hartmann, 2000) and animals (Matsuzaki *et al.*, 1982), the exact function of homospermidine is still uncertain.
It is suggested for phages that polyamines like spermidine are important for the DNA charge balance during DNA packaging (Yu & Schaefer, 2008). The negative charge of the DNA is shielded by the positive charge of the polyamine and therefore can be packed very compact.
Moreover, phage JG004 seems to be dependent on spermidine since a *P. aeruginosa speD* mutant which has a defect in spermdine synthesis (Heurlier *et al.*, 2006) can not be lysed by phage JG004 (data not shown).

Features of JG024 Genome

Phage JG024 is highly related to the widespread and conserved PB1-like viruses (Ceyssens *et al.*, 2009). The general characteristics as well as the similarity to the phage JG024 are shown in Table 15. The overall nucleotide similarity to the PB1-like phages varies between 86 % to phage PB1 and 95 % to the phages SN and 14-1 (Tab. 15). A phylogenetic tree showing the relation between these 6 closely related phages in comparison with the phage JG024 is presented in Fig. 11. The PB1-like phages reveal a very high sequence similarity and are distantly related to the *Burkholderia* phages Bcep1A and Bceo781 (Ceyssens *et al.*, 2009).

Table 15: Comparison of the JG024 genome with the PB1-like phages.

Phage	Genome size	GC content (%)	predicted ORFs	unique ORFs	DNA identity to JG024
JG024	66,275 bp	55.62	94	1	100%
PB1	65,764 bp	55.5	93	-	86 %
F8	66,015 bp	55.6	93	1	87 %
SN	66,390 bp	55.6	92	2	95 %
14-1	66,238 bp	55.6	90	-	95 %
LMA2	66,530 bp	55.6	95	2	93 %
LBL3	64,427 bp	55.5	88	2	92 %

The ability to infect a wide range of *P. aeruginosa* strains as described earlier (3.1.1) could be one reason for the success and wide distribution of this phage group. Moreover, the phage 14-1 which belongs to this highly conserved phage group is part of a clinical trial to treat *P. aeruginosa* infections with phage therapy. The phage was chosen for this trial due to its prevalence to clinical isolates (Merabishvili *et al.*, 2009). As described above, we could also show that phage JG024 is able to lyse several clinical isolates (3.1.1).

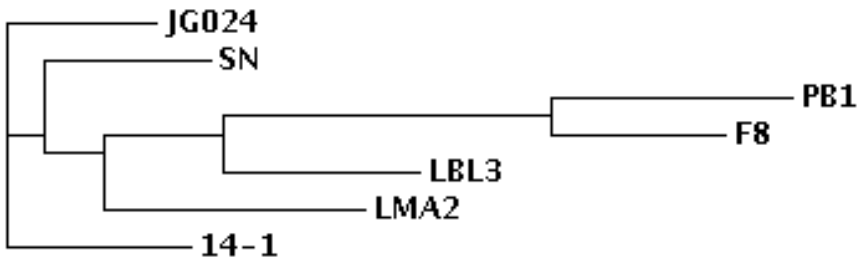

Figure 11: Phylogenetic tree of the phage JG024 and the PB1-like phages. This distance phylogram was made using the ClustalW online analysis tool. It shows the evolutionary relationship between JG024 and the other PB-1-like phages.

Since these phages have such a strong similarity a comparative ORF prediction was possible. The genome of JG024 is compact organized with only 7.1 % intergenic space. No RNA polymerase was detected suggesting that this phage uses the host transcriptional machinery, as it was also suggested for the PB1-like family of phages.

A terminase-like gene was found (ORF 4) at the beginning of the genome. The terminase is involved in DNA packaging as discussed for phage JG004. The terminase of phage JG024 shows 99.6 % identity to the terminase of phage SN.

We detected a putative structural gene cluster in the genome of JG024 which contains genes encoding for putative head structure proteins (ORF 18 and 19) as well as for tail and baseplate proteins (ORF 22-47). Since this phage is so similar to the PB1-like phages, it was possible to identify head and tail proteins as well as baseplate proteins. This was not possible for phage JG004 since this phage shows only similarities to probable tail proteins but not to head proteins. Moreover, ORF 40 was designated as a lytic tail protein. It was shown for the phages 14-1 and LBL3 that this protein has a transglycosylase domain with a N-acetyl-D-glucosamine binding site, which shows a specific degradation of peptidoglycan (Ceyssens *et al.*, 2009).

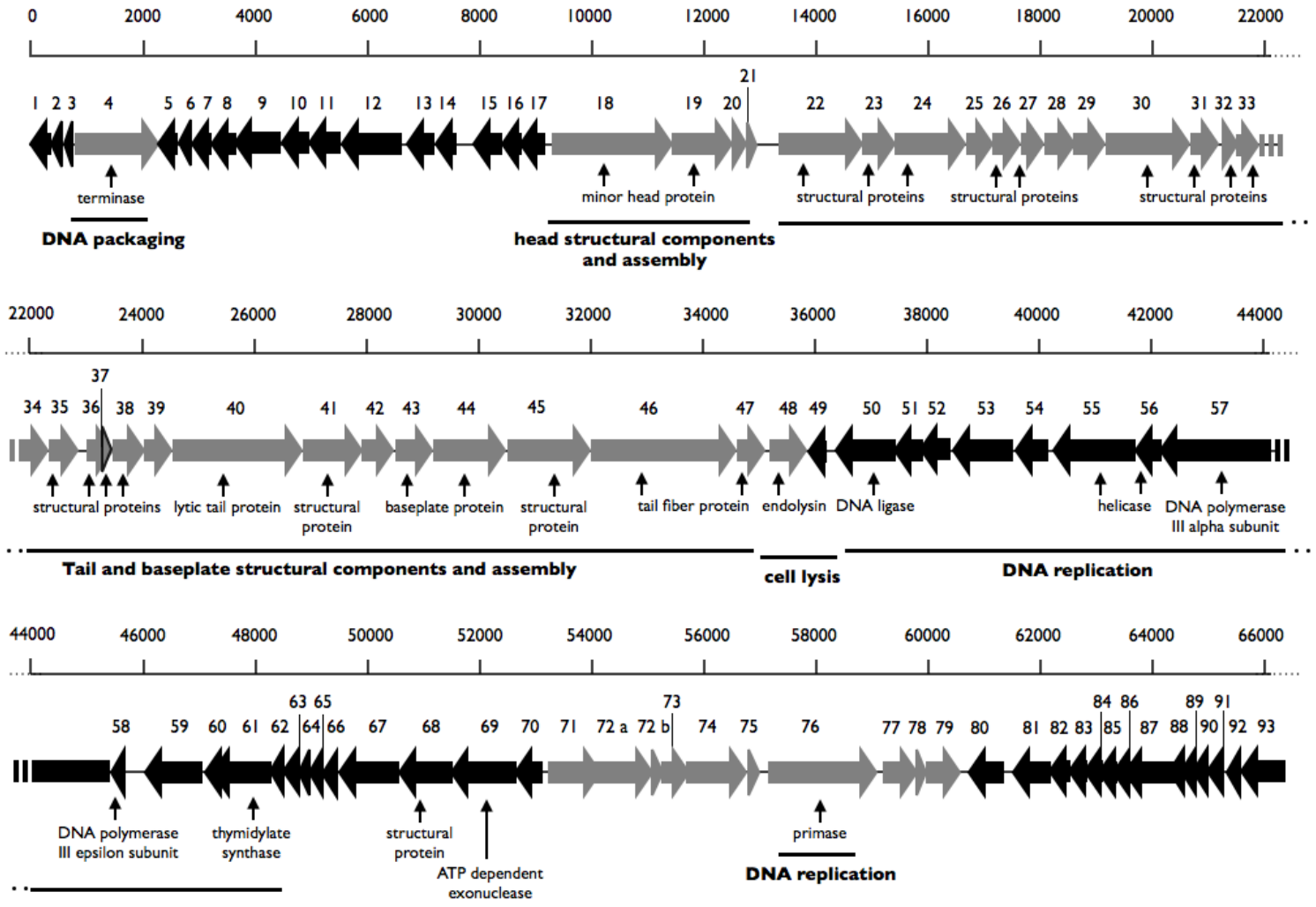

Figure 12: Schematic representation of the JG024 genome with its assumed ORFs and some functional assingments. The arrowheads point in the direction of transcription.

Moak & Molineux (2004) pointed out that enzymes with muralytic activity were found in diverse phages and that these enzymes are often associated with large proteins or structural components.

ORF 48 encodes for a putative endolysin with a high similarity to the endolysin of phage LMA2 (98.6 %) and contains a lysozyme-like superfamily. This protein accumulates probably in the cytoplasm since it lacks a signal peptide and a cell wall binding domain as it was shown for the PB1-like phages (Ceyssens *et al.*, 2009). Therefore, the endolysin is dependant on a holin which penetrates the inner membrane and facilitate access to the peptidoglycan layer (Wang *et al.*, 2000). ORF52 has 100 % identity to ORF 50 of phage F8 and to ORF 51 of phage 14-1. It was suggested for these phages that this ORF encodes for a probable holin since it is located near the endolysin, it has a small size (201 aa), and three transmembrane domains (Ceyssens *et al.*, 2009).

Moreover, a complete DNA replication machinery was detected suggesting that the DNA replication is host independent as it was described for the PB1-like phages. This gene cluster contains a DNA ligase (ORF 50), a helicase (ORF 55 and 56), a DNA polymerase III (ORF 57 and 58), as well as a thymidylate synthase (ORF 61). A putative primase was also found but is not included in this gene cluster (ORF 76). As shown for the other PB1-like phages (Ceyssens *et al.*, 2009) JG024 uses the DNA polymerase III, which is the main DNA replication enzyme in bacteria. The polymerase epsilon subunit which is encoded by the ORF 58 has a proofreading activity (Kelman & O'Donnell, 1995).

In contrast to phage JG004 no putative endonucleases or methyltransferases which are involved in DNA modification were detected. As described above (3.1.3) the DNA of phage JG024 was not cleaved by the restriction enzyme *Hind*III. Genome analysis revealed restriction sites for *Hind*III in the genome of JG024 suggesting that the DNA is modified. Since no sequence similarities for methyltransferases could be detected it is possible that there is a new not yet described or annotated

methyltransferase among the hypothetical proteins.

Several small genes encoding for hypothetical proteins were found. Two major clusters (ORF 1-17 (except ORF 4 which encodes for the terminase) and ORF 80-93) were identified. As discussed for phage JG004 these proteins may be involved as accessory factors during phage infection (Mann *et al.*, 2005).

Phage 14-1 (ORF 71) and phage LBL3 (ORF 68) own a hypothetical protein with a size of 434 aa. This protein is encoded by two ORFs in phage JG024, designated ORF 72 a (362 aa) and 72 b (60 aa). The two ORFs are seperated by only 116 bp. ORF 78 is a small predicted gene with a size of 132 bp and encodes for a unique protein in phage JG024. No similarities were found in databases. False annotation for this ORF was excluded since this ORF was identified by two ORF finder (GeneMark and ORF Finder) independently.

3.2 Impact of Antibiotics on *Pseudomonas aeruginosa* Lipopolysaccharide

As described in 1.3.2 the opportunistic human pathogen *Pseudomonas aeruginosa* exhibits various resistance mechanisms towards antibiotics. Several well-characterized factors as efflux pumps or an impermeable outer membrane are known to contribute to its intrinsic antibiotic tolerance (Strateva & Yordanov, 2009).
Antibiotic use can lead to different mutations in bacteria allowing adaptation to the antibiotic stress (Schurek *et al.*, 2008). Mutations in the LPS core biosynthesis cluster leading to a truncation of the LPS was observed in conjuction with antibiotic use before. Antibiotics like ciprofloxacin (Legakis *et al.*, 1989), gentamicin (Bryan *et al.*, 1984; Shearer & Legakis, 1985) and also carbenicillin (Godfrey *et al.*, 1984; Shearer & Legakis, 1985) were used in these studies suggesting that the altered LPS may impair the uptake of the antimicrobial compound. But still the exact reason for the alteration of the LPS as well as the exact mutation was not understood.
Since we isolated LPS specific phages we wanted to use these phages in a phage based *in vitro* system to investigate LPS mutations induced by antibiotic stress. These phages can serve as screening tools in random genomic mutagenesis experiments to identify mutations in the LPS or to monitor the emergence of LPS mutations in a culture under antibiotic pressure. To screen for *P. aeruginosa* LPS mutants the LPS specific phage JG004 was chosen due to its lytic life cycle and the low rate of spontaneous resistant bacteria occurring during infection (below 0.012 %).

3.2.1 Investigation of Clinical Isolates

Investigation of 23 *P. aeruginosa* clinical non-CF isolates revealed five mutants with altered LPS (Fig. 13). Compared to the LPS of a wild type strain as PAO1 the LPS of these clinical isolates is highly truncated. The thick lower band indicates the lipid A as well as the inner core polysaccharide of the LPS. Bands above this components represent the outer core polysaccharide and the O-antigen. Also, infection with the LPS specific phage JG004 was not possible suggesting that the LPS modification removed the receptor structures recognized by JG004 (3.1.2).

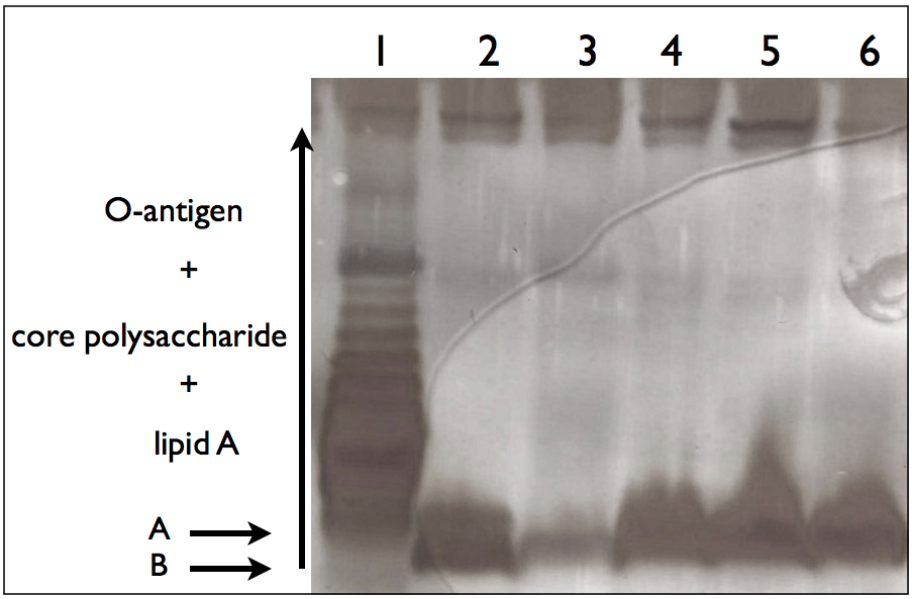

Figure 13: Silver stained 16.5 % tricine-SDS gel displaying the truncated LPS of five clinical isolates. The LPS was isolated as described in 2.9 and 3 μl of each LPS preparation was loaded to the gel. The arrows designated A and B indicate position of the wild type and truncated LPS, respectively. The LPS components of *P. aeruginosa* are indicated. The LPS is composed of the lipid A, the core polysaccharide and the O-antigen. 1, *P. aeruginosa* PAO1; 2, GH06 20934; 3, GH06 21466; 4, GH06 25135; 5, GH06 41910; 6, GH06 54773-2.

Moreover, all strains showed a high resistance to antibiotics (Tab. 16). Antibiotic susceptibility test stribes with 16 commonly used antibiotics were used and compared with the wild type PAO1.

Clearly, the clinical isolates showed increased tolerance to at least 3 antibiotic classes. Antibiotic classes like β-lactames (ampicillin, piperacillin), flourquinolones (ceftazidim, ciprofloxacin) as well as tetracyclines are substrates for the clinical relevant efflux pump *mexABoprM* (Schweizer, 2003). Ziha-Zarifi *et al.* (1999) showed that antimicrobial treatment of patients infected with *P. aeruginosa* leads to mutations in the regulator *mexR* resulting in an overexpressed *mexABoprM* operon. Further investigation of the efflux pump *mexABoprM* in these isolates revealed that at least one of the three repressors of the efflux pump, *mexR*, *nalC* or *nalD*, was

Table 16: Antibiotic susceptibility test. ATB PSE I test strips from BioMérieux were used as described in 2.5.7. S: sensitive (no growth); I: intermediate (growth at low antibiotic concentrations); R: resistant (growth at high antibiotic concentrations).

Antibiotic	Strain					
	PAO1	GH06 20934	GH06 21466	GH06 25135	GH06 41910	GH06 54773-2
Ampicillin + Sulbactam	R	R	R	R	R	R
Piperacillin	S	R	I	R	I	R
Piperacillin + Tazobactam	S	R	S	R	I	R
Ceftazidim	S	R	S	R	I	R
Aztreonam	I	R	I	R	R	R
Cefepin	S	R	S	R	I	R
Imipenem	S	R	R	S	S	S
Meropenem	S	R	I	I	S	I
Gentamicin	I	R	S	R	I	I
Tobramicin	S	I	S	I	S	S
Amikacin	S	I	S	I	S	I
Ciprofloxacin	S	R	R	R	S	R
Levofloxacin	S	R	R	R	I	R
Cotrimoxazol	R	R	R	R	R	R
Fosmomycin	R	R	S	S	R	S
Tetracyclin	R	R	R	R	R	R

mutated which finally results in an overexpression of the efflux pump. We amplified the *mexR*, *nalC* and *nalD* gene by PCR and sequenced the single genes as described in 2.8.1. Comparison with the wild type identified five point mutations in *mexR* of the isolate GH06 21466, one point mutation in *mexR* of the isolate GH06 20934 as well as ten point mutations in *mexR* of the isolate GH06 54773-2. The isolates GH06 25135 and GH06 41910 showed no mutations in *mexR* but both revealed two point mutations in *nalC* and one point mutation in *nalD*.
These results indicate that the clinical isolates are highly resistant to antibiotics due to the overexpressed efflux pump. However, a possible involvement of the truncated LPS in antibiotic resistance can not be excluded and was investigated in more detail.

3.2.2 *In vitro* Phage Based System to Detect LPS Alterations

A transposon mutagenesis using the mariner transposon was performed to verify that we were able to isolate LPS mutants by using the LPS specific phage JG004 as a screening tool. Moreover, this approach should provide mutants with highly truncated LPS comparable to the LPS of the clinical isolates. In addition, mutations of bacterial components essential for phage infection should also be identified. After the mutagenesis the bacteria were incubated with a tenfold excess of the LPS specific phage JG004. Bacteria with LPS modifications due to the insertion of the transposon were not lysed by the phage (Fig. 14).
Twenty three mutants were isolated and the genomic position of the mariner transposon was determined by arbitrary PCR (2.6.7) (Tab. 17). The LPS of the mutants was isolated and separated via tricine-SDS PAGE (2.9). It is clearly shown that the LPS profile of 15 mutants are different compared to the wild type PAO1 (Fig. 15). Truncated LPS was observed for the following mutants: *mexA* (TM2), *mexR* (TM5, TM8, and TM11), *nalD* (TM1, TM6 and TM10), *nalC* (TM9 and TM14), *wzz2* (TM12), *algC* (TM15), PA2200 (TM21), *rmlB* (TM20, TM22) and PA5000 (TM23). The transposon screen revealed genes important for LPS biosynthesis like the gene *algC* which is needed for complete LPS core biosynthesis in *P. aeruginosa* (Coyne *et al.*, 1994). Or the genes *rmlA* and *rmlB* which are involved in the biosyn-

Table 17: Identified transposon mutants. The mutants were screened with the phage JG004 and the integration of the transposon was identified by arbitrary PCR.

Transposon mutant	Integration of transposon
TM1, TM6, TM10	*nalD*
TM2	*mexA*
TM3	*speD*
TM4	PA0534
TM5, TM8, TM11	*mexR*
TM7	PA0421
TM9, TM14	*nalC*
TM12	*wzz2*
TM13	PA2555
TM15	*algC*
TM16	PA705
TM17	*rmlA*
TM18	*waaL*
TM19	PA5001
TM20, TM22	*rmlB*
TM21	PA2200
TM23	PA5000

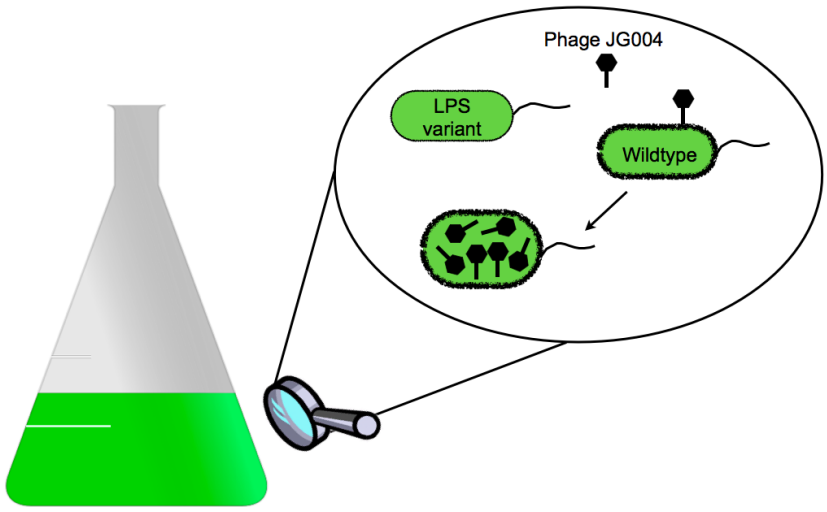

Figure 14: Scheme of *in vitro* phage based system to screen for LPS variants. The conical flask represents the mixture of *Pseudomonas aeruginosa* wild type and LPS variants. The LPS specific phage JG004 can only bind to the wild type but not to truncated LPS. JG004 will infect the wild type bacteria, propagate and finally lyse the cells.

thesis of the LPS core sugars (King *et al.*, 2009). However, we identified also 8 out of 23 mutants with LPS similar to the LPS of the wild type PAO1 (TM3 (*speD*), TM4 (PA0534), TM7 (PA0421), TM13 (PA2555), TM16 (PA0705), TM17 (*rmlA*) and TM18 (*waaL*) TM19 (PA5001)), suggesting that the transposon integrated in genes which are important for the phage infection cycle itself and not in a gene relevant for the receptor. The high amount of strains with truncated LPS (65 %) confirm that the screen is useful to isolate and identify LPS mutant.

But more interesting was the identification of the mutated regulators *mexR*, *nalC* and *nalD* among the transposon mutants. Nearly 35 % of the identified mutants have an integration in one of these regulators. Moreover, the LPS of these mutants appear very similar to the truncated LPS of the clinical isolates. A switch of of these regulators leads to the overexpression of the efflux pump *mexABoprM* (Srikumar *et al.*, 2000; Cao *et al.*, 2004; Sobel *et al.*, 2005). Since there are no re-

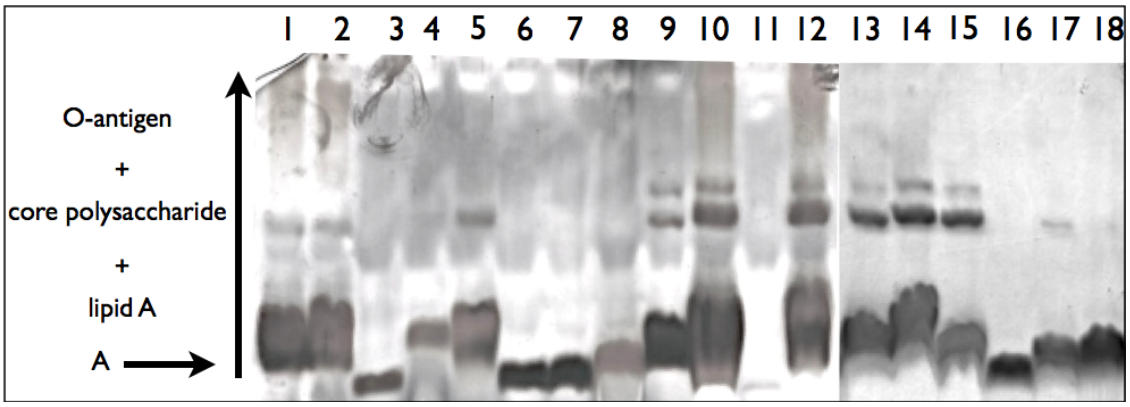

Figure 15: Silver stained 16.5 % tricine-SDS gel displaying the LPS profile of the isolated transposon mutants. The LPS was isolated as described in 2.9 and 3 μl of each LPS preparation was loaded to the gel. The arrow designated A indicate the position of the wild type LPS. The LPS components of *P. aeruginosa* are indicated. The LPS is composed of the lipid A, the core polysaccharide and the O-antigen. Distinct LPS truncation could be observed for the following *P. aeruginosa* transposon mutants: 3, TM2 (*mexA*); 6, TM5 (*mexR*); 7, TM6 (*nalD*); 8, TM14 (*nalC*); 11, TM15 (*algC*); 16, TM21 (PA2200); 17, TM 22 (*rmlB*); 18, TM23 (PA5000)). No or indistinct LPS modifications are shown for the the following samples: 1, PAO1; 2, *P. aeruginosa mexR* deletion mutant; 4, TM3 (*speD*); 5, TM4 (PA0534); 9, TM7 (PA0421); 10, TM13 (PA2555); 12, TM16 (PA0705); 13, TM17 (*rmlA*); 14, TM18 (*waaL*); 15, TM19 (PA5001).

ports about these regulators involved in LPS biosynthesis we constructed a *mexR* deletion mutant (2.7) and investigated the LPS. The LPS of JG09 shows no alteration (Fig. 15, Lane 2) as shown for the transposon mutants mentioned above. This result indicates that mutation of the gene *mexR* or the resulting overexpression of *mexABoprM* itself does not result in a modified LPS. This suggest that the truncated LPS seems to have its origin in a secondary mutation. Interestingly, we used two different antibiotics during the transposon screen, gentamicin and chloramphenicol (2.6.7). The resulting antibiotic stress is very high for the mutants, suggesting that secondary mutations leading to a better adaptation under these conditions are likely. For further experiments the transposon mutants TM5 and TM6 were used since both contained a highly truncated LPS and a transposon inserted in *mexR*

and *nalD* respectively.

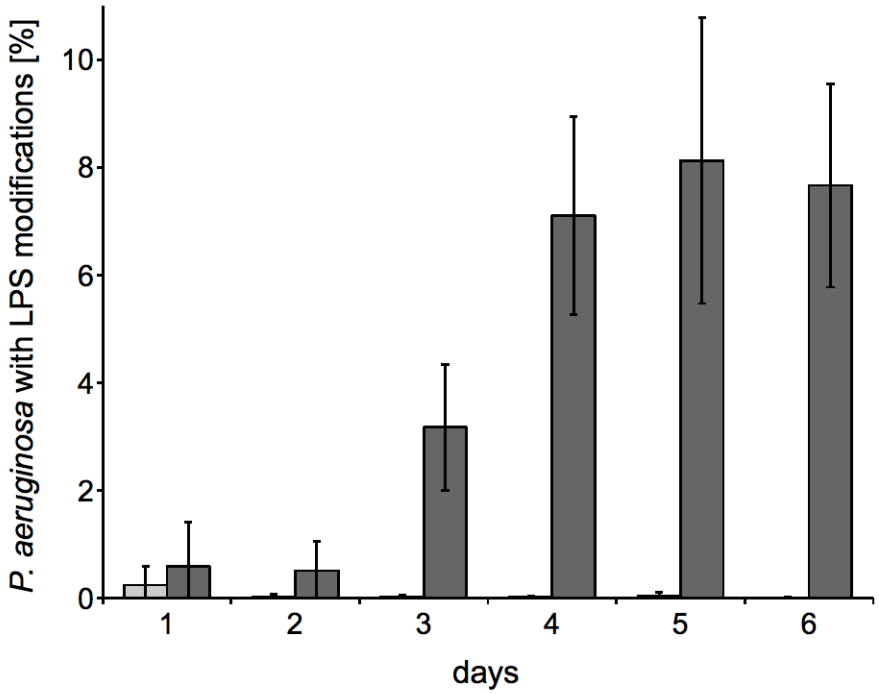

Figure 16: Emerging of *Pseudomonas aeruginosa* strains with altered LPS in the presence and absence of carbenicillin. The *P. aeruginosa mexR* deletion mutant was incubated in LB (light grey bars) and in LB with 200 μg/ml carbenicillin (dark grey bars) as described in 2.5.16. The experiment was carried out for 6 days. Each day, fresh medium was inoculated and the phage based *in vitro* system was used to monitor LPS alterations. Each bar indicates the determined percentage of mutants with truncated LPS per day. The rate of mutants with truncated LPS increases up to 8 % in the presence of carbenicillin.

3.2.3 Antibiotics Treatment Leads to Lipopolysaccheride Alterations

It is known that antibiotic treated *P. aeruginosa* overexpresses efflux pumps to exclude these substances out of the cell. The overexpression of the efflux pumps is due to mutations in the corresponding regulators (Poole, 2002). However, the involve-

ment of altered LPS in antibiotic resistance was discussed before (Legakis *et al.*, 1989; Bryan *et al.*, 1984; Shearer & Legakis, 1985; Godfrey *et al.*, 1984; Diver *et al.*, 1991; Abraham *et al.*, 2009) but the emergence of these mutants due to antibiotic treatment wasn't shown yet.

The phage based *in vitro* system (2.5.16) was used to monitor the emergence of mutants with truncated LPS during exposure to antibiotics. The *mexR* deletion mutant was used for these experiments because we found LPS mutations in the clinical isolates as well as in the transposon mutants always connected with an over-expressed efflux pump. Moreover, incubation of this mutant with antibiotics which are substrates for the efflux pump allowed a higher concentration of antibiotics in the culture.

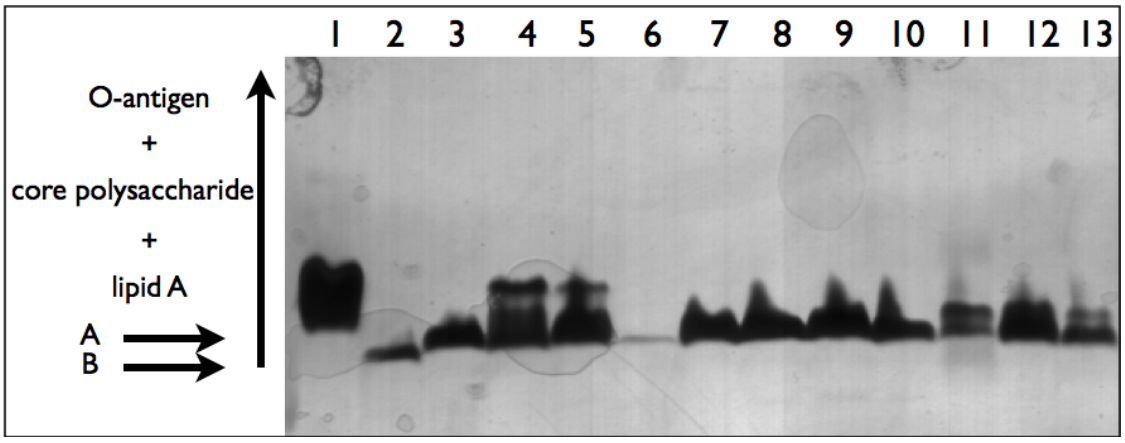

Figure 17: Silver stained 16.5 % tricine-SDS gel displaying isolated LPS of phage infection resistant *P. aeruginosa* clones. The clones were isolated from the phage based *in vitro* system to monitor antibiotic induced LPS modifications. All isolated clones display a modified LPS after antibiotic treatment. The LPS was isolated as described in 2.9 and 3 μl of each LPS preparation was loaded to the gel. The arrows designated A and B indicate the position of the wild type LPS. The LPS components of *P. aeruginosa* are indicated. The LPS is composed of the lipid A, the core polysaccharide and the O-antigen. The O-antigen structure cannot be observed in this gel due to short silver staining. 1, *P. aeruginosa* PAO1; 2-13, Isolated clones from the LPS assay.

Experiments were performed with the antibiotic carbenicillin which is a substrate of the *mexABoprM* efflux pump. To test whether the LPS structure changes during incubation with carbenicillin, we incubated the *mexR* deletion mutant in LB with and without the indicated antibiotic over 6 days. Each day we inoculated fresh medium and screened for LPS mutants with the LPS specific phage JG004. The resistance frequency of *P. aeruginosa* Δ*mexR* incubated with carbenicillin increased suddenly after 3 days to 4 % and reached a maximum of 8 % after 5 days (Fig. 16). In the absence of antibiotics the resistance frequency for *P. aeruginosa* Δ*mexR* is below 0.1 %. As a control we isolated 10 clones from this assay and investigated the LPS biochemically (2.9) (Fig. 17). Clearly, all mutants showed a truncated LPS. This experiment supports the hypothesis that LPS alterations emerge in response to antibiotic stress.

3.2.4 Antibiotics Induce Oxidative Stress

Recently, it was shown that the repressor MexR is able to sense oxidative stress (Chen *et al.*, 2008). The mechanism of this regulator is dependant on two cysteine residues.

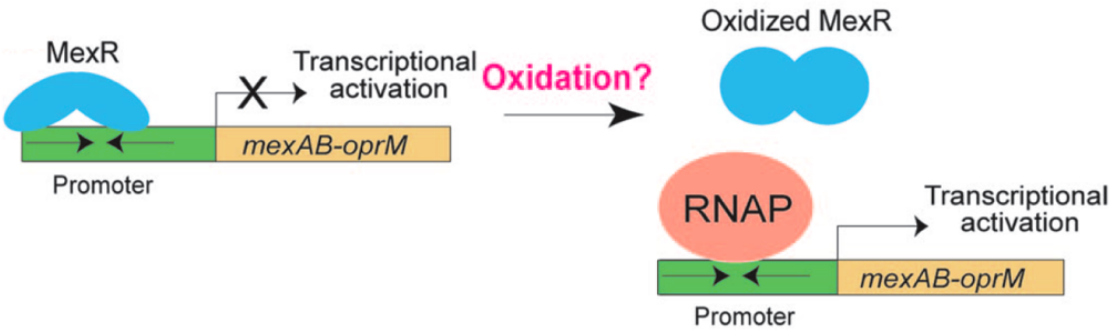

Figure 18: MexR senses oxidative stress. From Chen *et al.* (2008). MexR contains two cysteine residues which forms disulfide bonds under oxidizing conditions resulting in a dissociation of the regulator from the *mexR* DNA-recognition site.

These residues form under oxidizing conditions intramonomer disulfid bonds which lead to the dissociation of the regulator from the promotor DNA (Fig. 18). Moreover, Kohanski *et al.* (2007) pointed out that bactericidal antibiotics induce oxidative stress in the bacterial cell by inducing the so-called Fenton reaction. The mechanism is supposed to be a hyperstimulation of the electron transport chain. Due to this hyperstimulation single electrons are transferred to oxygen resulting in superoxid radical production. This radical damages iron sulfur clusters in the cell and allocate ferrous iron for the Fenton reaction. During the Fenton reaction the very reactive hydroxyl radical is produced which damages DNA, proteins and lipids in the cell.

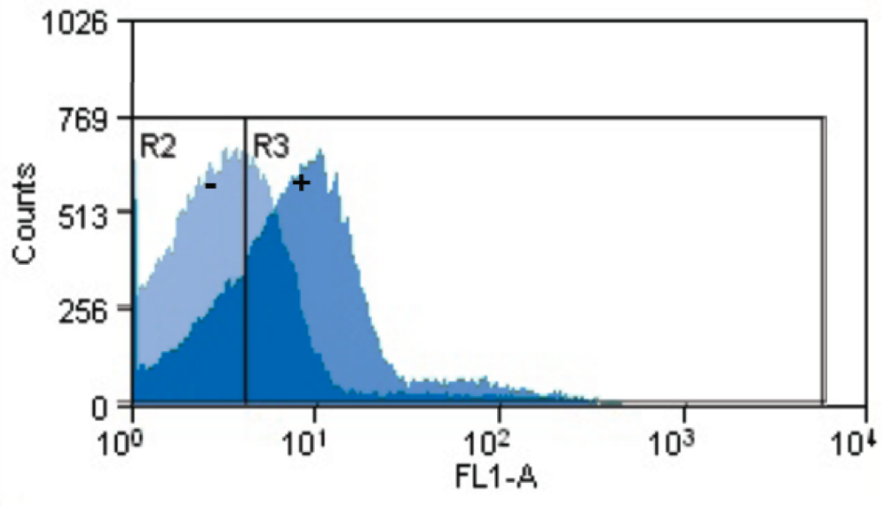

Figure 19: Reactive oxygen species assay of *Pseudomonas aeruginosa*. *P. aeruginosa* were grown in LB with (+) and without (-) gentamicin. The cells were flourescence stained with H_2DCFDA (2.5.17). The x-axis describes the flourescence intensitiy of the cells and the y-axis the cell count.

Since these expermiments were performed in *E. coli* the same experiment was done in *P. aeruginosa* in this work to see if antibiotics induce reactive oxygen species (ROS) and hence induce oxidative stress in a similar way. During this experiment the cells were first incubated with carbenicillin, gentamicin or menadione as described in 2.5.17 and then incubated with the fluorescence dye H_2DCFDA. This dye

penetrates the bacterial cell and is non-flourescent until it becomes first deacetylated by intracellular esterases and finally oxidized by radicals. The cleavage of lipophilic groups by the esterase results in a charged form of the dye which is better retained by the cells (Rosenkranz *et al.*, 1992). The flourescence was measured by fluorescence activated cell sorting (FACS). Carbenicillin, gentamicin and as a control the superoxid radical producer menadione were used. All three substances induced reactive oxygen species. Fig. 19 shows the FACS results for cells treated with gentamicin and cells untreated. Clearly, the flourescence intensity and the corresponding amount of reactive oxygen species is higher in cells treated with gentamicin. The same results were obtained with carbencillin and menadione and are not shown.

3.2.5 Mutants with Altered LPS are More Tolerant to Oxidative Stress

I found the emergence of *P. aeruginosa* mutants with truncated LPS when cultures were treated with the antibiotic carbenicillin (3.2.3) and showed that antibiotics induce oxidative stress (3.2.4). The next step was to investigate if the altered LPS contributes to the tolerance to oxidative stress. To trigger oxidative stress in *P. aeruginosa* we used menadione as a superoxide radical producer. Survival assays were done with different concentrations of menadione as described in 2.5.8. The transposon mutants TM5 and TM6 as well as the clinical isolate GH06 41910 and the wild type PAO1 were used.

The transposon mutants TM5, TM6 and the clinical isolate showed an increased survival in the presence of oxidative stress compared with the wild type PAO1 suggesting that the modified LPS of the three strains contributes to the tolerance (Fig. 20). MIC determinations with menadione and H_2O_2 also showed that the LPS alteration has an impact on the resistance of the cell to oxidative stress since strains with truncated LPS were more tolerant to these substances (Data not shown).

If these mutants have a higher tolerance to oxidative stress than the wild type, the rate of LPS mutants should increase in an environment with increased oxidative stress. It was shown that intracellular free ferrous iron increases the production of the hydroxyl radical by induction of the Fenton reaction (Keyer & Imlay, 1996).

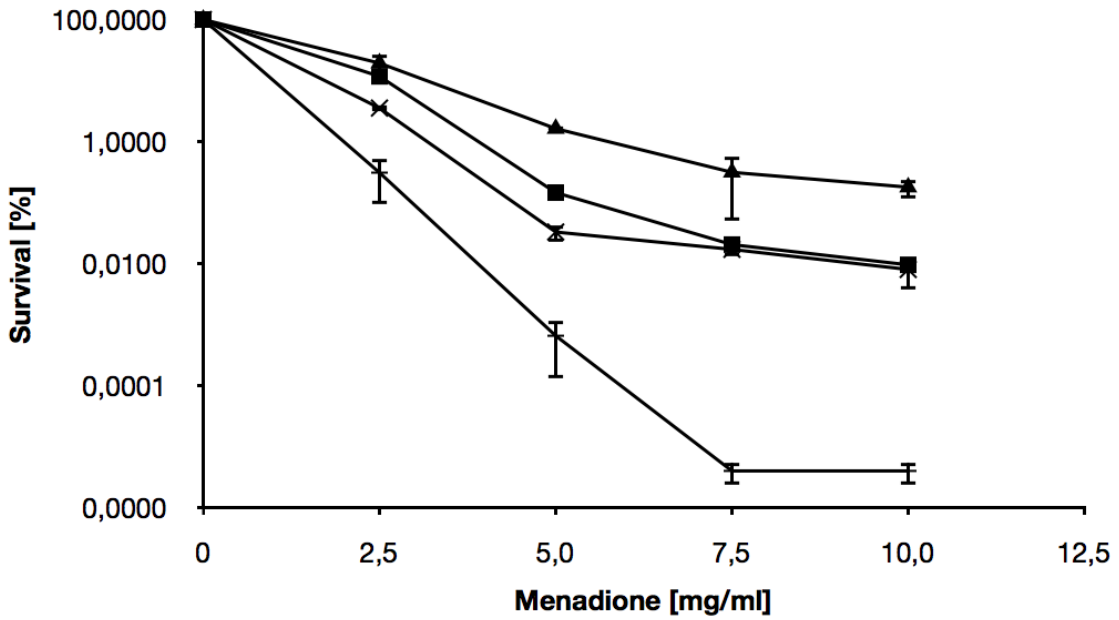

Figure 20: Survival of different *Pseudomonas aeruginosa* strains challenged with menadione. *P. aeruginosa* PAO1 (dash), the *P. aeruginosa* transposon mutants TM5 (crosses), TM6 (triangles) and the clinical isolate GH06 41910 (squares) after exposure to increasing concentrations of menadione. Incubation time was 4 h at 37 ℃ with a menadione concentration range of 2.5 mM to 10 mM. After incubation at 37 ℃ the colony forming unit was determined by serial dilutions as described in 2.5.4 to calculate the survival rate in percent.

Since ferrous iron can increase the oxidative stress in a culture, LB without ferrous iron and LB with 1 mM ferrous sulfate to prevent and increase the Fenton reaction and therefore hydroxyl radical production in the cell was used (2.5.16). The *mexR* deletion mutant was incubated with and without carbenicillin for 6 days and inoculated every day into fresh medium as described earlier (3.2.3). In LB medium without ferrous iron the Fenton reaction is dramatically reduced. As a result no mutants with a truncated LPS were observed (data not shown). On the contrary, Δ*mexR* incubated in LB with 1 mM ferrous sulfate and carbenicillin leads to an

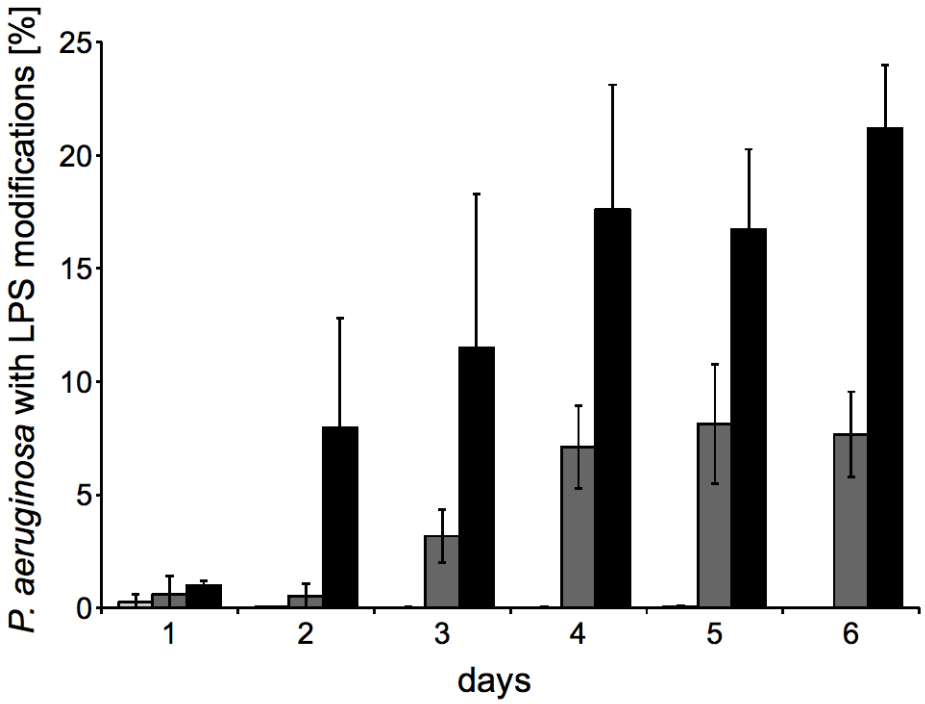

Figure 21: Emerging of *Pseudomonas aeruginosa* strains with altered LPS in the presence and absence of carbenicillin and ferrous sulfate. The *P. aeruginosa mexR* deletion mutant was incubated in LB (light grey bars), in LB with 200 μg/ml carbenicillin (dark grey bars) (Fig. 16) and in LB supplemented with 200 μg/ml carbenicillin and 1 mM ferrous sulfate (black bars) as described in 2.5.16 to induce the Fenton reaction. The experiment was carried out for 6 days. Each day, fresh medium was inoculated and the phage based *in vitro* system was used to monitor LPS alterations (2.5.16). Each bar indicates the determined percentage of mutants with truncated LPS per day. The rate of mutants with truncated LPS increases up to 8 % in the presence of carbenicillin and up to 22 % in the presence of carbenicillin and ferrous sulfate.

dramatic increase of LPS mutants up to 22 % (Fig. 21). Without ferrous sulfate the rate of LPS mutants reached a value of only 8 % (Fig. 21). To summarize these results, we could show that antibiotics induce the emergence of LPS modification in *P. aeruginosa* and that the induction of oxidative stress by ferrous sulfate increase the rate of LPS modifications suggesting that LPS modifications occur due to oxidative stress induced by antibiotics. High oxidative stress leads to high amounts of LPS alterations in the population.

3.2.6 PA5001 is Responsible for the LPS Alterations during Antibiotic Exposure

To identify the mutated gene which leads to the LPS modification we performed a complementation experiment with a *P. aeruginosa* cosmid library as described in 2.6.8. To screen for mutants which gained wild type LPS due to complementation, a phage was isolated which is specific to the truncated LPS of the mutants.

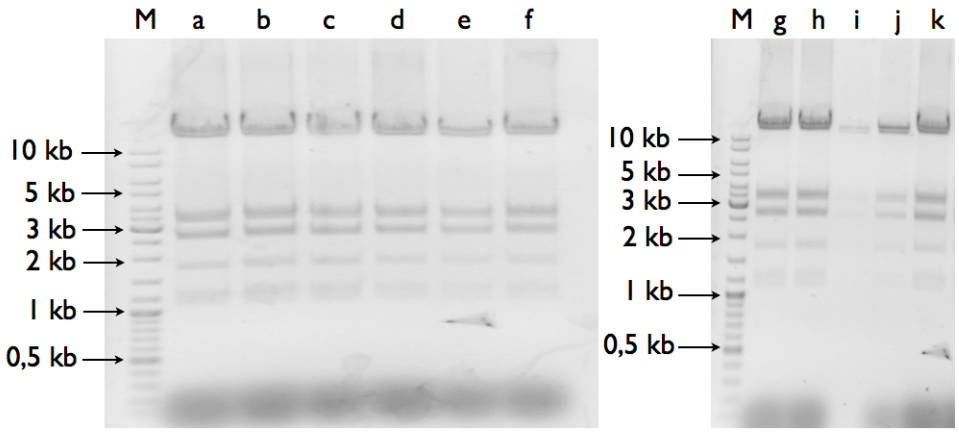

Figure 22: Restriction pattern of isolated cosmids from eleven complemented clones of the *Pseudomonas aeruginosa* mutant TM5 (a-k). The eleven clones were isolated after the cosmid library screen and showed sensitivity to the phage JG004 infection, which requires wild type LPS. Shown are 1 % agarose gels after staining with ethidiumbromide. Cosmid was digested with *Eco*RI and *Hind*III. M: Molecular weight marker.

The phage SZ01 was able to infect the described LPS mutants including the presented clinical isolates but not the PAO1 wild type. Altogether we could identify 11 complemented clones. The clones were resistant to the phage SZ01 and contained LPS similar to the wild type PAO1 (data not shown). Moreover, the clones were sensitive to the LPS specific phage JG004 after complementation. The cosmid was isolated and analyzed by digestion with restriction enzymes (2.6.8). An identical restriction pattern indicates that all clones were complemented by the same cosmid (Fig. 22).

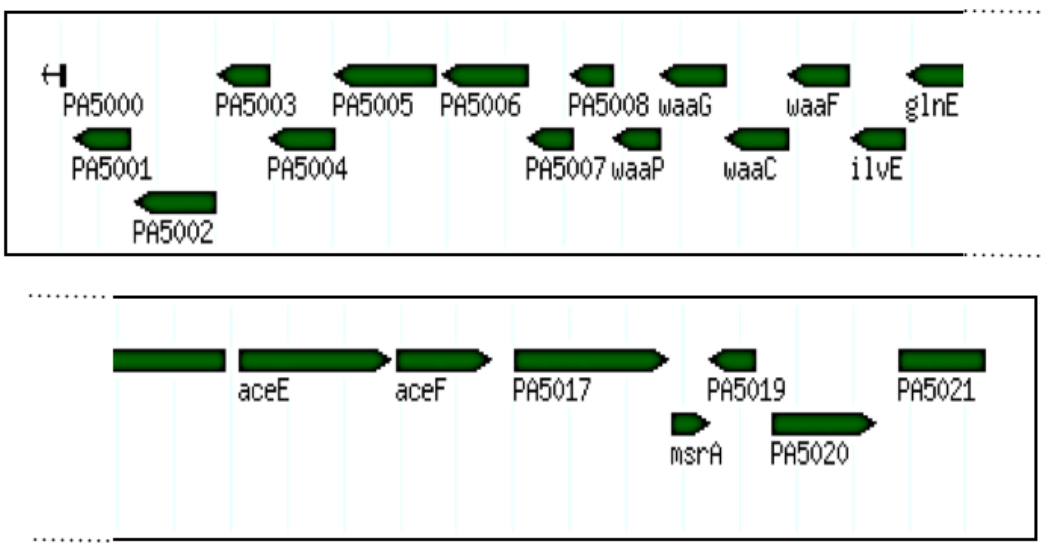

Figure 23: Genomic organization of cosmid insert. The insert has a size of ca. 31 kb covering the region from 5618374 to 5649292 of the *Pseudomonas aeruginosa* PAO1 genome. Arrows indicate length and orientation of genes. Altogether the cosmid insert contains 20 complete genes of the *P. aeruginosa* PAO1 genome (provided by www.pseudomonas.com)

To identify the genes of the cosmid insert, sequencing analysis was done using primers binding to the flanking regions of the multiple clonig site present in cosmid pLAFR3 (2.6.8). The sequencing results were compared with the www.pseudomonas.

com genome database. The cosmid harbors a 31 kb insert covering a region starting at 5618374 to 5649292 of the *P. aeruginosa* genome. The first 13,3 kb including the genes PA5001 to *waaF* (PA5012) are part of a probable LPS core biosynthesis cluster (Fig. 23).

To identify the defect gene in the LPS mutants, each gene of this operon was amplified by PCR and cloned into a pUCP20T vector for complemen- tation experiments. Finally, we could identify PA5001 which allowed complementation of LPS mutants. PA5001 is a uncharacterized probable glycosyltransferase (King *et al.*, 2009). Glycosyltransferases are known to transfer monosaccharides to an acceptor molecule. Complementation with the gene PA5001 leads to wild type LPS in the transposon mutants TM5 and TM6 and also in the clinical isolate GH06 41910 (Fig. 24). Moreover, it was not possible to complement the other four clinical isolates with this gene, but sequence analysis showed several mutations in PA5001.

To confirm our results we constructed a PA5001 deletion mutant and a *mexR*PA5001 double deletion mutant (2.7). The PA5001 single mutant and the *mexR*PA500 double mutant have a similar LPS pattern as the clinical isolates and the transposon mutants (Fig. 24). Complementation of the PA5001 and *mexR*PA5001 deletion mutant with PA5001 restored wild type LPS (Fig. 24).

To further confirm the oxidative stress resistance phenotype observed in mutants TM5, TM6 and GH06 41910 (3.2.5) we challenged the PA5001 and *mexR*PA5001 mutants with menadione as described (2.5.8). Fig. 25 clearly shows that the tolerance to menadione in the knockout mutants is comparable to the phenotype of the transposon mutants and the clinical isolate. PA5001 restored sensitivity to menadione, as observed for the wild type, in the deletion mutants (Fig. 25) as well as in the transposon mutants and the clinical isolate GH06 41910 (Fig. 26). This indicates that mutations in PA5001 result in a higher tolerance to oxidative stress. Since the double mutant *mexR*PA5001 displays the same phenotype as the PA5001 single mutant, the gene *mexR* seems to play no role in this oxidative stress experiment. Moreover, the MIC for three antibiotics was determined to see if the LPS truncation contributes to high level antibiotic resistance. I used the antibiotics carbenicillin, levofloxacin and nalidixic acid and tested the MIC for the wild type PAO1 and the

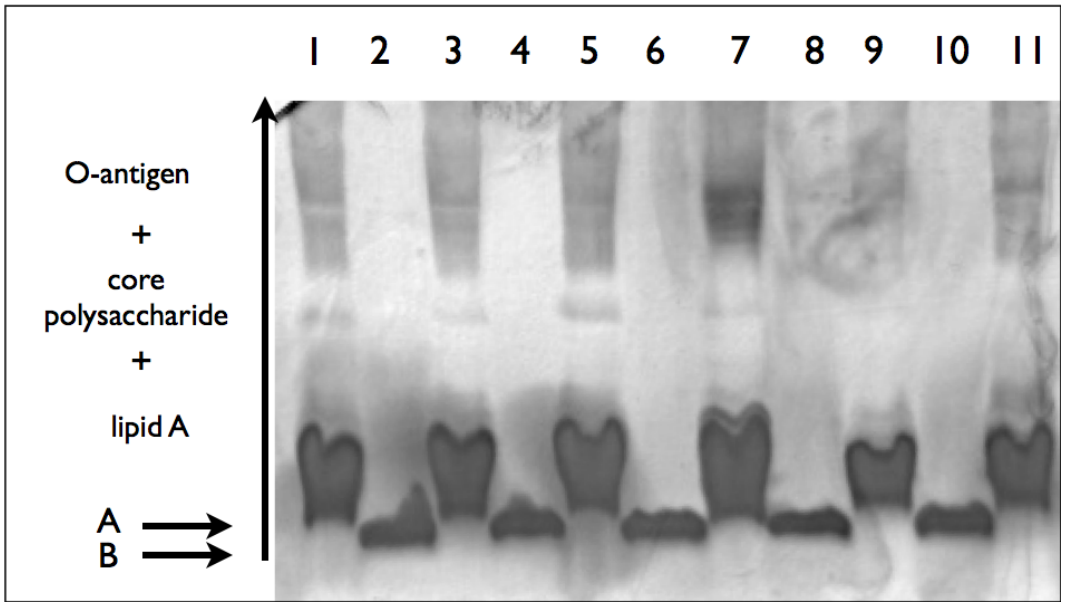

Figure 24: Silver stained 16.5 % tricine-SDS gel displaying the truncated LPS after complementation with PA5001 using the plasmid pJG004. Complementation with the gene PA5001 restored wild type LPS. The LPS was isolated as described in 2.9 and 3 μl of each LPS preparation was loaded to the gel. The arrows designated A and B indicate the position of the wild type and truncated LPS, respectively. The LPS components of *P. aeruginosa* are indicated. The LPS is composed of the lipid A, the core polysaccharide and the O-antigen. The LPS of the following *P. aeruginosa* strains are shown: 1, PAO1; 2, TM5; 3, TM5 complemented with PA5001; 4, TM6; 5, TM6 complemented with PA5001; 6, GH06 41910; 7, GH06 41910 complemented with PA5001; 8, PA5001 deletion mutant; 9, PA5001 deletion mutant complemented with PA5001; 10, *mexR*PA5001 deletion mutant; 11, *mexR*PA5001 deletion mutant complemented with PA5001.

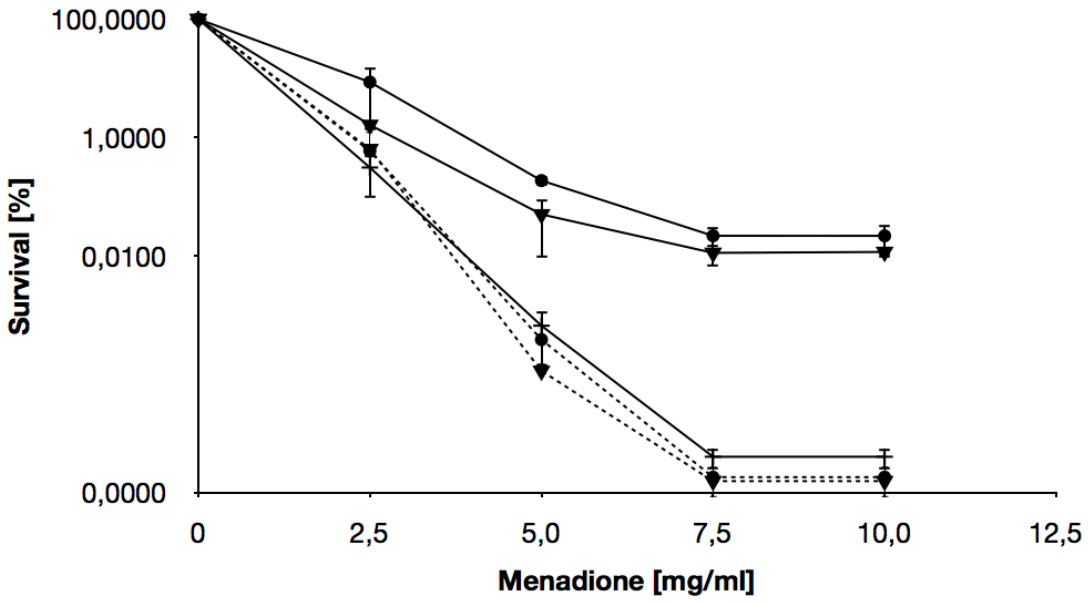

Figure 25: Survival of different *Pseudomonas aeruginosa* strains challenged with menadione. *P. aeruginosa* PAO1 (dash), the *P. aeruginosa* PA5001 (circles) and the *mexR*PA5001 (inverted triangles) deletion mutant after exposure to increasing concentrations of menadione. Incubation time was 4 h at 37 ℃ with a menadione concentration range of 2.5 mM to 10 mM. After incubation at 37 ℃ the colony forming unit was determined by serial dilutions as described in 2.5.4 to calculate the survival rate in percent. Complementation with PA5001 is represented by the dashed lines

PA5001 deletion mutants as described in Material and Methods (2.5.6). Fig. 27 shows that the MIC for the PA5001 mutant is slighlty increased for all three tested antibiotics compared to the wild type. These results and the results above indicate that a mutation in PA5001 has only a marginal effect on the resistance to antibiotics. But indicates a significant contribution to the survival during oxidative stress.
The gene PA5001 was also found in the work of Schurek *et al.* (2008) in connection with aminoglycoside resistance. A PA14 transposon library was used in this approach to identify mutants with an increased tobramicin resistance. Mutation in PA5001 was identified to play a role in low-level tobramicin resistance. Since

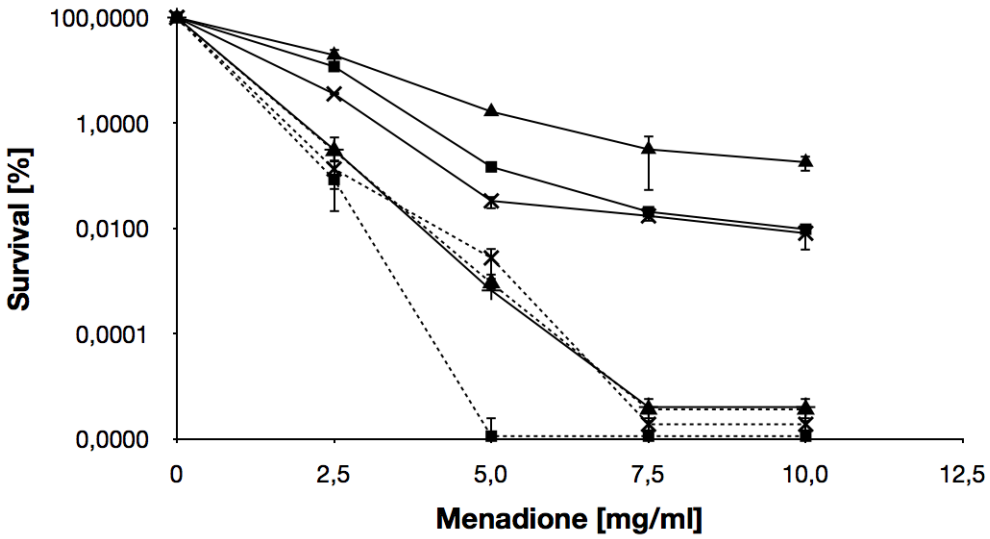

Figure 26: Survival of different *Pseudomonas aeruginosa* strains challenged with menadione. *P. aeruginosa* PAO1 (dash), the *P. aeruginosa* transposon mutants TM5 (crosses), TM6 (triangles) and the clinical isolate GH06 41910 (squares) after exposure to increasing concentrations of menadione. Incubation time was 4 h at 37 ℃ with a menadione concentration range of 2.5 mM to 10 mM. After incubation at 37 ℃ the colony forming unit was determined by serial dilutions as described in 2.5.4 to calculate the survival rate in percent. Complementation with PA5001 is represented by the dashed lines

mutations in PA5001 occur in the presence of antibiotics we suggest that the secondary mutations of the transposon mutants in PA5001 are due to the high antibiotic concentrations used during the transposon screen.

Moreover, our experiments revealed no direct connection between the mutation in *mexR* and PA5001. Both mutations are likely to emerge independently since both mutations have an impact on the resistance to antibiotics. One mutation leads to the export of antibiotics and the other to a tolerance to oxidative stress which damages important cell molecules like the DNA, proteins and also lipids. However, we found a new mechanism of antibiotic resistance which involves unexpected mutations of the probable glycosyltransferase PA5001. This finding may play a significant role in

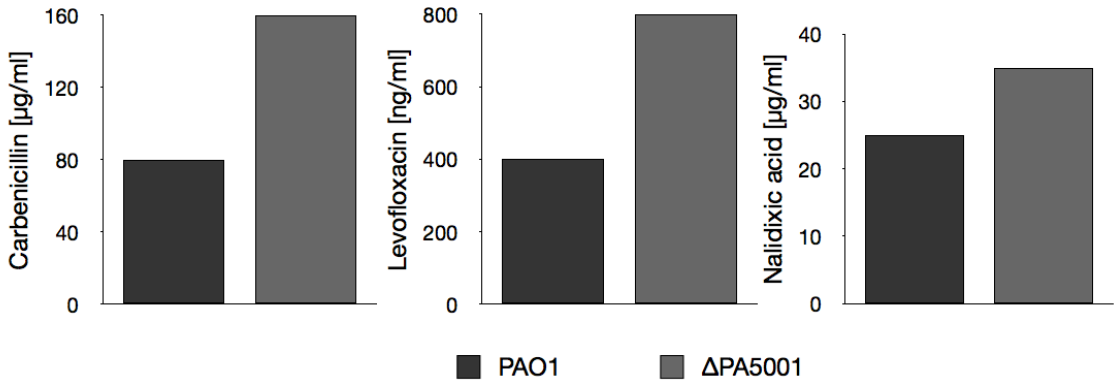

Figure 27: Minimal inhibitory concentration (MIC) determinations of the wild type PAO1 and the PA5001 deletion mutant. The antibiotics carbenicillin, levofloxacin and nalidixic acid were used as described in 2.5.6.

the specific adaptation to antibiotic stress and increases our knowledge of antibiotic resistance mechanisms in *P. aeruginosa*.

3.2.7 Involvement of OprI in oxidative stress tolerance

I could show that antibiotic treatment leads to mutations in the gene PA5001 which results in the truncation of the LPS. This truncation increases the tolerance to oxidative stress. However, the exact mechanism behind the tolerance is unknown. It was shown before that the lipoprotein OprL of *P. aeruginosa* helps protect the cell from oxidative stress (Panmanee *et al.*, 2008). Therefore, we isolated the proteins from the outer membrane as described in 2.10 to investigate if the truncated LPS has an impact on the outer membrane proteins and if these proteins contribute to the oxidative stress tolerance.

The isolated proteins of the wild type PAO1, the *mexR* as well as the *mexR*PA5001 and PA5001 deletion mutants were separated via SDS PAGE (Fig. 28). Clearly, the profiles of the outer membrane proteins look similar exept for a stronger signal of a band with a size of around 9 kDa in the *mexR*PA5001 and PA5001 deletion mutants. Mass spectrometry revealed the presence of lipoprotein OprI in this protein band which is encoded by the gene PA2853. OprI is a small protein of 8.83

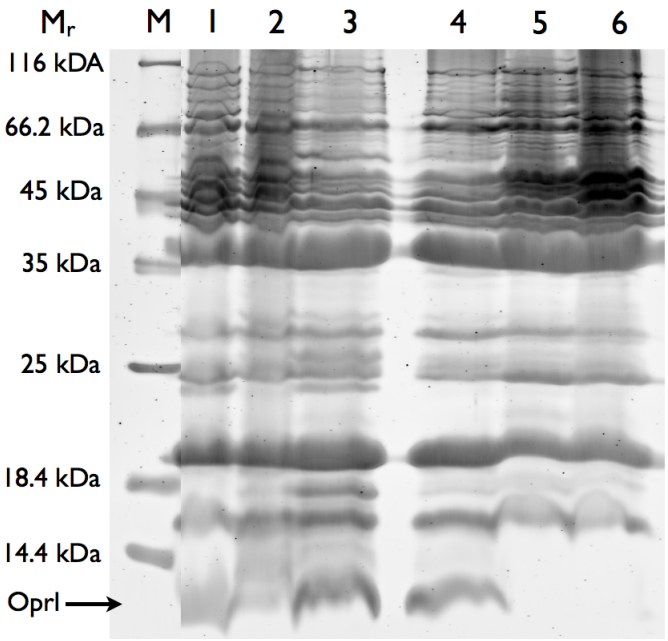

Figure 28: SDS-PAGE (15 %) of isolated outer membrane proteins. Outer membrane proteins were isolated, separated and stained as described in 2.10. 50 μg protein was loaded to the gel for each sample. The arrow indicates the position of the lipoprotein OprI with a size of approximately 9 kDa. M, Molecular weight marker; 1, PAO1; 2, *P. aeruginosa mexR* deletion mutants; 3, *P. aeruginosa* PA5001 deletion mutant; 4, *P. aeruginosa mexR*PA5001 deletion mutant; 5, *P. aeruginosa* PA5001 deletion mutant complemented with PA5001; 6, *P. aeruginosa mexR*PA5001 deletion mutant complemented with PA5001.

kDa which corresponds to the determined molecular mass of the protein band with a size of approximately 9 kDa in Fig. 28. OprI is one of the major proteins of the outer membrane in *P. aeruginosa* and probably functions as a physical link between the outer membrane and the peptidoglycan (Duchêne *et al.*, 1989). This protein is highly conserved among different *P. aeruginosa* strains and therefore used as an antigen for possible vaccines (Bumann *et al.*, 2010).

Interestingly, complementation of PA5001 in the PA5001 and *mexR*PA5001 mutant leads to complete loss of the corresponding protein band (Fig. 28). This indicates that the truncation of the LPS leads to higher amounts of OprI in the outer mem-

brane and it is possible that this protein contributes to the oxidative stress tolerance as it was shown for the lipoprotein OprL. Still, the exact mechanism which helps to understand the observed tolerance is not completely understood. This mechanism involves a mutation in PA5001 which probably has other effects like the impact on the amount of OprI and therefore making this mechanism to a complicated but very interesting research object.

4 Outlook

The genome sequence and the comprehensive characterization of both phages opens several interesting questions, which should be addressed in future studies.
Both phages may be suitable for phage therapy, especially their ability to lyse alginate overproducing clinical isolates, rewards further research. It should be tested if these phages contain alginate degrading enzymes on their surface which would facilitate infection of mucoid strains.
Moreover, the low similarity to other phages makes JG004 an interesting research topic. Only few genes encoding for proteins of the phage particle were found. Isolation of the phage capsid and characterization of these proteins would help to find the corresponding genes. A probable endolysin was detected in JG004 due to sequence similarities which has to be proved experimentally. Finally, the dependence of phage JG004 on spermidine produced by the host bacterium has to be investigated in more detail.

We could show that mutations in the uncharacterized probable glycosyltransferase PA5001 emerge due to antibiotic stress. Unexpectedly, the mutation helps protect the cells from oxidative stress which are induced by antibiotics. Still, the interesting mechanism behind the tolerance is unknown and has to be investigated. The possible involvement of the lipoprotein OprI in this mechanism was discussed but is not completely understood yet. A gene deletion of the corresponding gene would unravel the involvement of OprI. Further research could also address the cytotoxicity of the truncated LPS.

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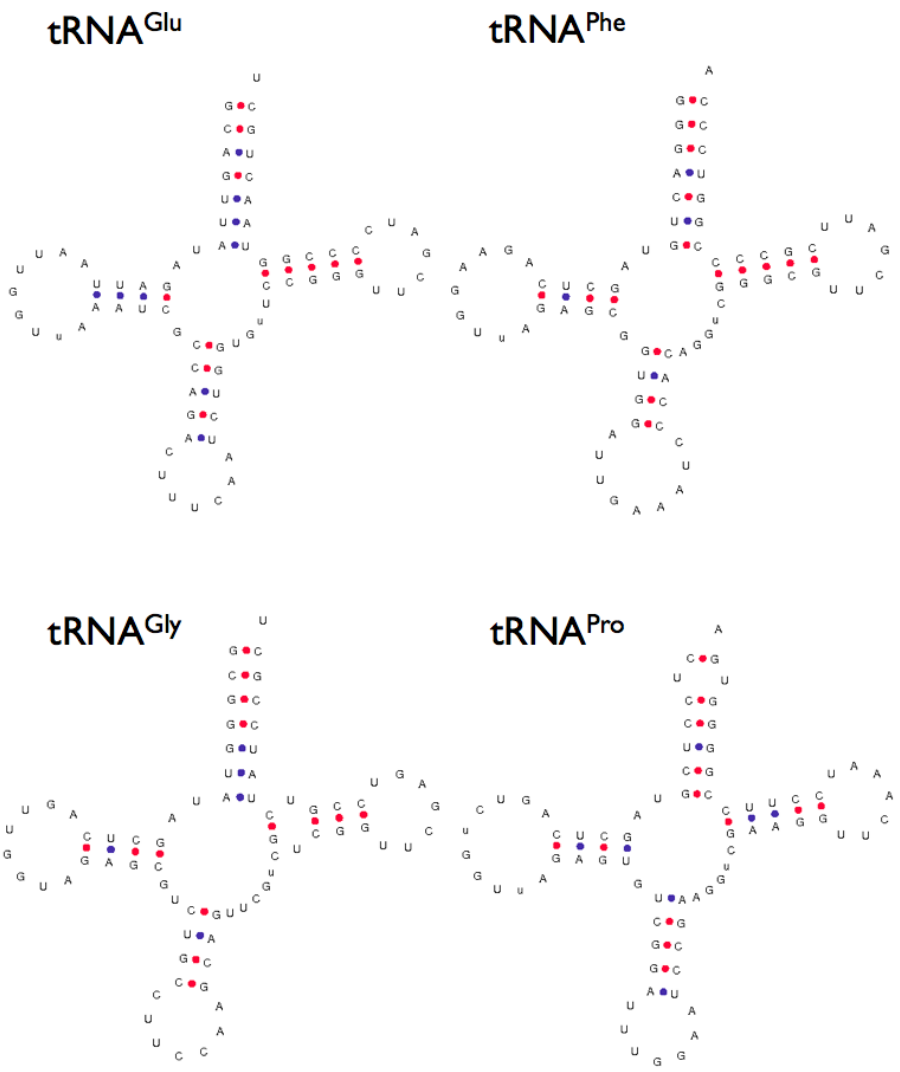
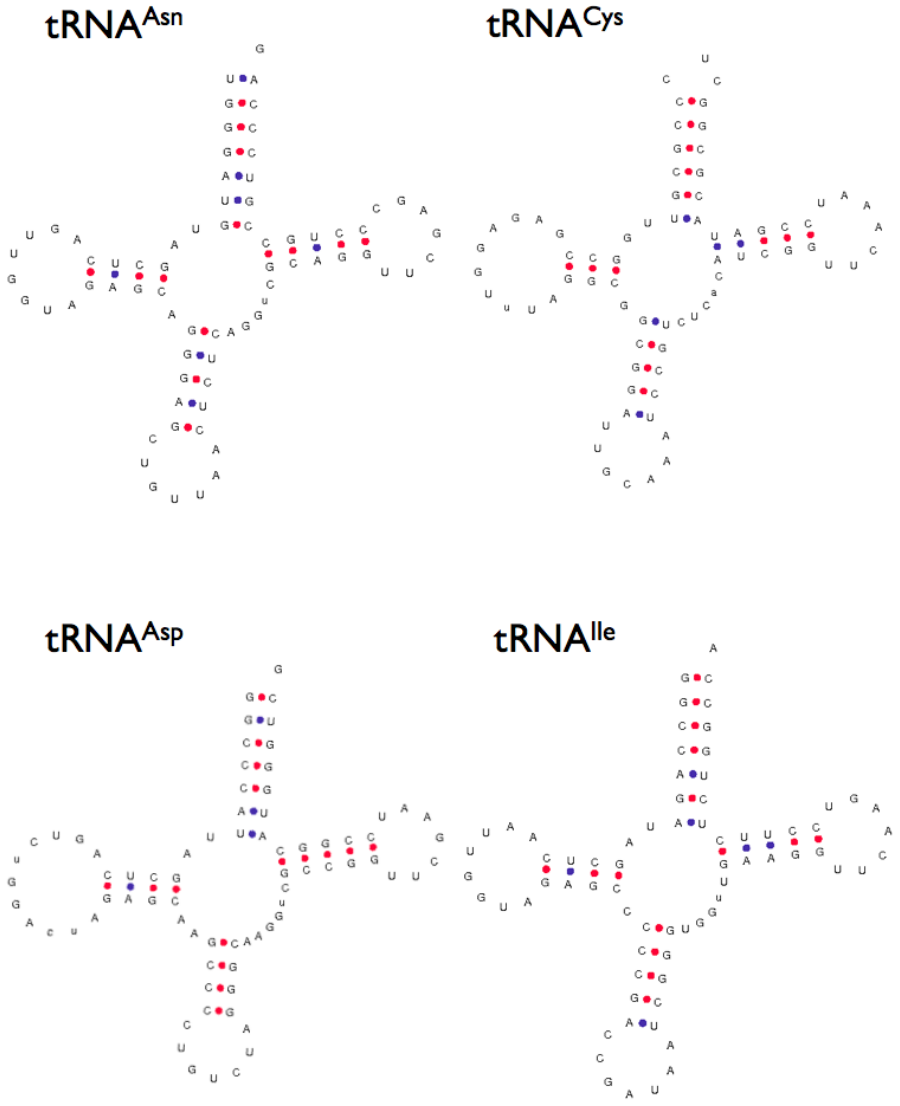
Appendix

tRNAs of JG004




tRNA^Asn
tRNA^Cys
tRNA^Asp
tRNA^Ile

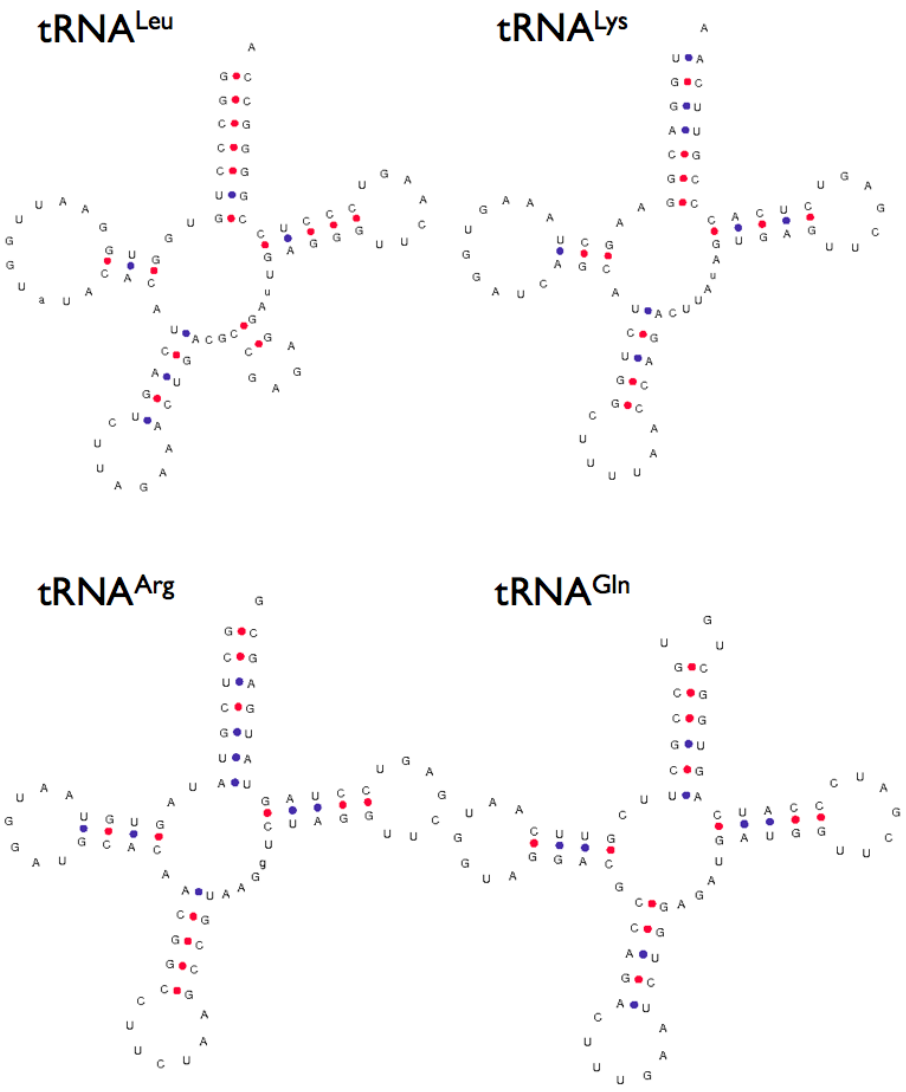


Figure 29: The sequence of the 12 tRNAs of JG004 in cloverleaf structure.

Table 18: Codon Usage of *P. aeruginosa*, phage JG004 and JG024. * = stop codon.

Codon	Amino acid	*P. aeruginosa* frequency/acid (%)	JG004 frequency/acid (%)	JG024 frequency/acid (%)
GCU	alanin	4.14	40.3	16.98
GCG	alanin	33.41	12.48	27.4
GCC	alanin	58.27	23.16	41.73
GCA	alanin	4.17	24.06	13.89
AUC	isoleucine	90.87	52.5	68.47
AUA	isoleucine	2.27	8.11	8.13
AUU	isoleucine	6.86	39.39	23.4
UAU	tyrosine	20.78	40.48	52.22
UAC	tyrosine	79.22	59.52	47.78
UGU	cysteine	10	36.36	23.53
UGC	cysteine	90	63.64	76.47
CAU	histidine	28.95	39.58	35.39
CAC	histidine	71.05	60.42	64.61
GUU	valine	3.94	29.81	21.12
GUG	valine	48.49	22.57	25.61
GUC	valine	41.78	20.04	39.96
GUA	valine	5.78	27.58	13.32
CGU	arginine	10.41	22.29	12.87
CGG	arginine	18.49	14.78	17.13
CGC	arginine	64.72	14.78	42.56
CGA	arginine	3.11	17.11	12.79
AGG	arginine	2.64	13.16	7.6
AGA	arginine	0.62	17.89	7.05
UAG	*	11.45	8.13	6.38
UAA	*	9.45	55	43.62
UGA	*	79.1	36.86	50
UGG	tryptophan	100	100	100
CAG	glutamine	85.3	48.11	67.51
CAA	glutamine	14.7	51.89	32.49

Codon	Amino acid	*P. aeruginosa* frequency/acid (%)	JG004 frequency/acid (%)	JG024 frequency/acid (%)
AAU	asparagine	14.18	35.75	32.4
AAC	asparagine	85.82	64.25	67.57
UCU	serine	1.51	25.25	14.13
AGU	serine	4.77	15.05	8.10
UCG	serine	23.66	15.11	23.87
UCC	serine	21.89	15.05	26.17
AGC	serine	47.11	24.31	22.16
UCA	serine	1.06	5.23	5.58
AAA	lysine	12.53	41.64	36.37
AAG	lysine	87.47	58.36	63.63
CUU	leucine	2.49	19.37	12.01
UUG	leucine	7.03	14.26	15.31
UUA	leucine	0.23	2.79	1.49
CUG	leucine	66.77	29.08	44.84
CUC	leucine	22.36	15.88	22.13
CUA	leucine	1.13	18.63	4.22
CCU	proline	4.18	37.49	18.68
CCG	proline	65.77	28.24	52.44
CCC	proline	25.76	13.44	14.65
CCA	proline	4.29	20.84	14.23
GAU	aspartic acid	19.70	42.56	44.08
GAC	aspartic acid	80.3	57.44	55.92
ACU	threonine	3.97	36.12	23.73
ACG	threonine	15.24	12.4	20.15
ACC	threonine	78.84	27.09	48.96
ACA	threonine	1.95	24.39	7.16
UUU	phenylalanine	4.85	23.46	15.74
UUC	phenylalanine	95.15	76.54	84.26
GAG	glutamic acid	61.46	51.79	43.85
GAA	glutamic acid	38.54	48.21	56.15
AUG	methionine	100	100	100
GGU	glycine	9.81	29.27	16.93
GGG	glycine	11.75	16.45	13.39
GGC	glycine	73.51	28.6	52.48
GGA	glycine	4.93	25.68	17.2

Table 19: The genes of phage JG004 and their predicted function. HP = Hypothetical protein.

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
1	96	224	129	42	HP	HP of *Enterobacteria* phage WV8 (20/36)	2.0
2	295	543	249	82	HP		
3	540	827	288	95	HP		
4	827	1138	312	103	HP		
5	1338	1811	474	157	HP (DUF335)	HP*Enterobacteria* phage RB43 (32/37)	$9e^{-10}$
6	1837	2001	165	55	HP		
7	2440	2613	174	57	HP		
8	2836	3822	987	328	HP	HP *Clostridium difficile* (67/233)	$1e^{-9}$
9	3991	4242	252	83	HP		
10	4254	4493	240	79	HP		
11	4495	4689	195	64	HP		
12	4699	5202	504	167	HP		
13	5222	5488	267	88	HP		
14	5677	5991	315	104	HP		
15	5991	6227	237	78	HP		
16	6227	6460	234	77	HP		
17	6460	6708	249	82	HP		
18	6905	8650	1746	581	putative ribonucleotide reductase (RNR PFL)	NrdA *Aeromonas* phage 31 (211/567)	$3e^{-100}$
19	8643	9689	1047	348	HP (Ferritin like)	HP *Pseudomonas* phage PA11 (118/314)	$4e^{-52}$
20	9719	10063	345	114	HP	HP *Enterobacteria* phage Felix01 (32/83)	

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
21	10066	11013	948	315	putative thymidylate synthase (Thy1)	thymidylate synthase *Escherichia phage* rv5 (158/325)	$6e^{-77}$
22	11220	12170	951	316	HP	HP *Burkholderia ambifaria* phage BcepF1 (41/76)	
23	12163	12375	213	70	HP		
24	12379	12714	336	111	HP		
25	12733	12939	207	68	HP		
26	12967	13149	183	60	HP	HP *Burkholderia oklahomensis* EO147 (24/56)	0.9
27	13146	13925	780	259	HP	constituent protein *Pseudomonas* phage PaP3 (99/235)	$7e^{-44}$
28	13922	14173	252	83	HP		
29	14106	14276	171	56	HP		
30	14273	14710	438	145	HP		
31	14707	14913	207	68	HP		
32	14934	15332	399	132	HP	HP *Psychroflexus torquis* (33/127)	0.001
33	15329	15892	564	187	HP	HP *Enterobacteria* phage phiEcoM-GJ1 (61/171)	$2e^{-20}$
34	15889	16941	1053	350	putative exodeoxyribonuclease	putative exodeoxyribonuclease *Escherichia* phage rv5 (102/374)	$1e^{-30}$
35	16983	17204	222	73	HP		
36	17214	17447	234	77	HP		
37	17517	18521	1005	334	HP		
38	18623	19339	717	238	HP		
39	19537	19935	399	132	HP		

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
40	20025	22739	2715	904	putative DNA polymerase (DNA pol A)	DNA polymerase *Escherichia* phage rv5 (152/548)	$1e^{-44}$
41	22800	24653	1854	617	putative DNA primase/helicase (TOPRIM; P loop NTPase)	primase/helicase *Roseobacter* phage SI01 (140/508)	$9e^{-41}$
42	24715	24901	186	61	HP		
43	24898	25143	246	82	HP		
44	25619	25915	297	98	HP		
45	26012	26314	303	100	HP		
46	26317	26481	165	54	HP		
47	26468	27124	657	218	HP (PRK11409)	HP *Rhizobium etli* 8C-3 (26/82)	0.013
48	27121	27663	543	180	HP	HP *Ralstonia pickettii*12J (24/86)	0.004
49	27802	28287	486	161	HP		
50	28299	28496	198	65	HP		
51	28497	28691	195	64	HP		
52	28804	28947	144	47	HP		
53	28937	29170	234	77	HP		
54	29208	29441	234	77	HP		
55	29472	30608	1137	378	putative RNA ligase	putative RNA ligase *Pseudomonas* phage 20phi2-1 (151/466)	$1e^{-44}$
56	30611	30766	156	51	HP		
57	30759	31568	810	269	HP	gp06 *Burkholderia* phage BcepMu (58/100)	$1e^{-22}$
58	31570	31890	321	106	HP		
59	31901	32209	309	102	HP		

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
60	32605	32910	306	101	HP	HP *Pseudomonas aeruginosa* PACS2 (20/49)	1.1
61	32900	33031	132	43	HP		
62	33048	33485	438	145	HP	HP *Clostridium sp.* (33/134)	0.33
63	33472	33711	240	79	HP		
64	33729	34289	561	186	putative peptidoglycan binding protein	putative peptidoglycan binding protein *Pseudomonas aeruginosa* (101/189)	$2e^{-50}$
65	34306	35805	1500	499	HP	phage protein *Enterobacteria* phage phiEcoM-GH1 (32/88)	$4e^{-6}$
66	35819	36193	375	124	putative tail fiber protein	putative tail fiber protein prophage *Pseudomonas syringae* (23/65)	0.005
67	36237	38294	2058	685	putative tail fiber protein	putative tail fiber protein *Aggregatibacter* phage S1249 (36/98)	$3e^{-6}$
68	38305	39036	732	243	HP	HP *Haemophilus* phage Aaphi23 (41/110)	$2e^{-12}$
69	39055	40518	1464	487	putative baseplate component	putative baseplate component *Erwinia* phage phiEa21-4 (128/481)	$7e^{-31}$
70	40520	40891	372	123	HP	HP Iodobacteriophage phiPLPE (38/108)	$5e^{-10}$
71	40902	41642	741	246	putative baseplate protein (DUF586)	putative baseplate protein *Haemophilus* phage Aaphi23 (39/129)	$3e^{-8}$
72	41639	42556	918	305	HP	HP *Pseudomonas fluorescens* PF0-1 (117/304)	$3e^{-54}$
73	42553	42909	357	118	HP	HP *Ralstonia pickettii* 12D (21/89)	0.024
74	42915	43676	762	253	HP	HP *Pseudomonas fluorescens* PF0-1 (90/231)	$1e^{-39}$
75	43673	46039	2367	788	putative tape measure protein (COG3941)	tape measure protein *Burkholderia* phage BcepNazgul (69/217)	$8e^{-19}$
76	46036	46188	153	50	HP		

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
77	46296	46667	372	123	HP		
78	46681	47160	480	159	HP	HP *Kingella oralis* ATCC 51147 (48/145)	$1e^{-7}$
79	47160	47633	474	157	HP	*Haemophilus influenzae* 86-028NP (36/96)	$2e^{-13}$
80	47735	48259	525	174	HP	HP *Bordetella petrii* DSM 12804 (51/146)	$3e^{-13}$
81	48272	49048	777	258	putative endonuclease (GIY-YIG)	endonuclease SegD *Vibrio* phage KVP40 (50/110)	$4e^{-12}$
82	49107	50393	1287	428	putative structural protein	structural protein *Erwinia* phage phiEa21-4 (132/450)	$3e^{-39}$
83	50406	50969	564	187	putative methyltransferase	cysteine methyltransferase *Geobacillus* sp. G11MC16 (32/104)	0.23
84	50966	51346	381	126	HP	HP *Haemophilus influenzae* 22.4-21 (37/119)	0.015
85	51346	51759	414	137	putative RNA polymerase	putative RNA polymerase *Xanthomonas* phage OP2 (44/130)	$1e^{-4}$
86	51797	52273	477	158	HP	HP *Erwinia* phage phiEa21-4 (42/129)	$1e^{-12}$
87	52323	53357	1035	344	HP	bacteriophage-related protein *Rhodobacter sphaeroides* (116/345)	$4e^{-41}$
88	53402	53812	411	136	HP		
89	53840	54757	918	306	HP	HP *Escherichia* phage rv5 (31/111)	$1e^{-6}$
90	54754	55224	471	156	putative methyltransferase	N6 adenine-specific DNA methyltransferase *Haliangium ochraceum* (45/137)	$4e^{-10}$
91	55234	56673	1440	479	HP (DUF935)	HP *Enterobacteria* phage WV8 (191/486)	$6e^{-102}$

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
92	56686	58206	1521	506	putative terminase (COG5362)	putative terminase *Erwinia* phage phiEa21-4 (191/458)	$2e^{-83}$
93	58328	58609	282	93	HP		
94	58811	59110	300	99	HP		
95	59485	59589	105	34	HP		
96	60645	60827	183	60	HP		
97	60897	61220	324	107	HP		
98	62012	62479	468	155	HP	aminotransferase *Rhodobacterales* bacterium HTCC2654 (21/71)	0.023
99	62476	62832	357	118	HP	HP *Pseudomonas aeruginosa* PA14 (28/108)	0.017
100	62880	63428	549	182	putative protease subunit (Clp protease like)	ClpP ATP-dependent protease subunit *Escherichia* phage rv5 (52/161)	$1e^{-9}$
101	63489	63674	186	61	HP		
102	63671	63964	294	97	HP	Orf41 *Pseudomonas* phage D3 (35/53)	$2e^{-12}$
103	64031	64463	432	143	HP		
104	64460	64738	279	92	HP	HP *Campylobacter gracilis* RM3268 (21/58)	0.27
105	64752	65066	315	104	HP		
106	65068	65727	660	219	putative HNH endonuclease (AP2)	HNH endonuclease Bacteriophage T5 (39/92)	$6e^{-7}$
107	65736	66005	270	89	HP		
108	66002	66214	213	70	HP	HP *Enterobacteria* phage EcoDS1 (28/69)	$2e^{-8}$
109	66224	66460	237	78	HP		

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
110	66463	66879	417	138	putative deoxycytidylate deaminase (cytidine deaminase like)	deoxycytidylate deaminase *Bacteroides vulgatus* (54/123)	$7e^{-20}$
111	66876	68084	1209	402	putative DNA ligase (DNA ligase AM)	ATP-dependent DNA ligase *Escherichia* phage rv5 (128/417)	$9e^{-32}$
112	68097	68561	465	154	HP		
113	68619	69179	561	186	putative cell wall hydrolase (Hydrolase 2)	cell wall hydrolase SleB *Methylobacterium populi* BJ001 (59/134)	$3e^{-18}$
114	69181	69741	561	185	putative phosphohydrolase	Metal dependent phosphohydrolase *Lentisphaera araneosa* (51/151)	$9e^{-11}$
115	69731	70162	432	143	putative hydrolase	hydrolase protein *Cardiobacterium hominis* (14/22)	0.069
116	70162	70716	555	184	putative phosphoesterase (PP2Ac)	phosphoesterase *Chryseobacterium gleum* (76/188)	$3e^{-29}$
117	70729	70965	237	78	HP		
118	70967	71242	276	91	HP (COG5606)	HP *Kingella oralis* (21/58)	$2e^{-5}$
119	71239	71646	408	135	HP	HP *Enterobacteria* phage JS98 (46/161)	$9e^{-5}$
120	71658	72575	918	305	putative RNA ligase/tail attachment protein (RNA lig T4 1)	RNA ligase 1 and tail attachment protein *Escherichia* phage rv5 (120/304)	$3e^{-43}$
121	72586	73005	420	139	HP	HP *Escherichia* phage rv5 (54/133)	$3e^{-21}$
122	73015	73890	876	291	putative ribose-phosphate pyrophosphokinase (Pribosyltran)	putative ribose-phosphate pyrophosphokinase *Enterobacteria* phage Felix 01 (97/264)	$4e^{-34}$
123	73945	74163	219	72	HP		

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
124	74162	75850	1689	562	putative nictotinate phospho-ribosyltransferase (PRTase type II)	putative nictotinate phosphoribosyltransferase *Enterobacteria* phage WV8 (238/594)	$4e^{-99}$
125	75862	76059	198	65	HP		
126	76056	76535	480	159	HP (Macro Poa1p like)	HP *Acinetobacter* phage 133 (66/160)	$2e^{-21}$
127	76547	76696	150	49	HP		
128	76880	77587	708	235	HP	HP *Aeromonas* phage 25 (79/194)	$5e^{-29}$
129	77664	77975	312	103	HP		
130	77962	78465	504	167	HP	HP *Pseudomonas fluorescens* Pf0-1 (44/144)	$3e^{-10}$
131	78467	78808	342	113	HP (P-loop NTPase)		
132	79052	79438	387	128	HP	HP *Pseudomonas* phage LIT1 (31/117)	0.017
133	79438	79749	312	103	HP		
134	79737	80015	279	92	HP		
135	80018	80719	702	233	HP		
136	80736	81158	423	140	HP		
137	81148	81552	405	134	putative homospermidine synthase	homospermidine synthase (34/101) *Cupriavidus taiwanensis*	0.32
138	81552	81818	267	88	HP		
139	81872	82030	159	52	HP		
140	82307	82450	144	47	HP		
141	82550	83203	654	217	HP	HP *Listonella* phage phiHSIC (49/162)	$2e^{-6}$

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
142	84381	84575	195	64	HP	HP LMA2 (59/64)	$5e^{-27}$
143	84673	84939	267	88	HP		
144	85342	85731	390	130	HP		
145	86047	86328	282	93	HP		
146	86328	86807	480	159	HP	HP *Proteus vulgaris* (35/108)	$6e^{-5}$
147	86942	87421	480	159	HP	HP *Pseudomonas* phage 201phi2-1 (37/123)	$4e^{-4}$
148	87457	88098	642	213	HP		
149	88121	88702	582	193	HP	HP *Erwinia* phage phiEa21-4 (66/193)	$9e^{-14}$
150	88817	89209	393	130	HP		
151	89206	89472	267	88	HP		
152	89505	89729	225	74	HP		
153	89726	90022	297	98	HP		
154	90040	90234	195	64	HP		
155	90231	90503	273	90	HP		
156	90500	90868	369	122	HP		
157	90868	91206	339	112	HP		
158	91211	91834	624	207	HP	HP Cyanophage P-SSP7 (92/233)	$3e^{-40}$
159	91958	92341	384	127	HP		
160	92334	92633	300	100	HP		

Table 20: The genes of phage JG024 and their predicted function. HP=hypothetical protein.

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
1	25	327	303	100	HP	HP of phage 14-1 (96/100)	$6e^{-48}$
2	383	556	174	57	HP	HP of phage SN (56/57)	$3e^{-25}$
3	607	753	147	48	HP	HP of phage SN (48/48)	$5e^{-17}$
4	802	2313	1512	503	terminase, large subunit (DUF1545; terminase 3)	terminase of SN (458/460)	0.0
5	2350	2733	384	127	HP	HP of phage 14-1 (127/127)	$7e^{-67}$
6	2730	2948	219	72	HP	HP of phage 14-1 (72/72)	$4e^{-35}$
7	2948	3298	351	106	HP	HP of phage LBL3/14-1 (106/106)	$2e^{-54}$
8	3344	3745	402	133	HP	HP of phage 14-1 (132/133)	$7e^{-69}$
9	3748	4527	780	259	HP	HP of phage 14-1 (259/259)	$1e^{-151}$
10	4614	5111	498	165	HP	HP of phage SN (143/145)	$2e^{-75}$
11	5068	5655	588	195	HP	HP of phage LBL3/14-1 (195/195)	$6e^{-113}$
12	5757	6689	933	310	HP	HP of phage 14-1 (310/310)	0.0
13	6792	7292	501	166	HP (NADB Rossmann)	HP of phage 14-1 (165/165)	$3e^{-93}$
14	7312	7659	348	115	HP	HP of phage 14-1 (114/115)	$5e^{-59}$
15	7908	8453	546	181	HP	HP of phage F8 (153/181)	$8e^{-84}$
16	8425	8748	324	107	HP	HP of phage SN (105/107)	$4e^{-57}$
17	8780	9181	402	133	HP	HP of phage LMA2 (133/133)	$8e^{-72}$
18	9362	11659	2298	765	minor head protein (Phge rel HI1409; DUF2212)	minor head protein phage 14-1 (748/765)	0.0
19	11659	12495	837	278	putative minor head protein (Phage Mu F)	putative minor head protein phage LMA2 (268/278)	$3e^{-157}$
20	12515	12721	207	68	HP	HP of phage LBL3 (68/68)	$2e^{-31}$

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
21	12718	12858	141	46	HP	HP of phage SN (44/46)	$2e^{-16}$
22	13370	14806	1437	478	putative structural protein (DUF2213)	putative structural protein phage LMA2 (461/478)	0.0
23	14810	15445	636	211	putative structural protein	structural protein phage SN (211/211)	$1e^{-115}$
24	15455	16603	1149	392	putative major structural protein	putative major structural protein phage LMA2 (379/382)	0.0
25	16705	17142	438	145	HP	HP of phage SN (143/145)	$8e^{-80}$
26	17157	17624	468	155	putative structural protein	putative structural protein phage LMA2 (155/155)	$1e^{-85}$
27	17648	18019	372	123	putative structural protein (PHA02310)	structural protein phage SN (123/123)	$8e^{-66}$
28	18027	18578	552	183	HP	HP of phage LMA2 (183/183)	$2e^{-104}$
29	18575	19156	582	193	HP	HP of phage F8 (193/193)	$3e^{-107}$
30	19260	20687	1428	475	putative structural protein	putative structural protein phage PB1 (468/475)	0.0
31	20746	21198	453	150	putative structural protein	putative structural protein phage LMA2 (147/150)	$2e^{-79}$
32	21294	21521	228	75	putative structural protein	putative structural protein phage LMA2 (75/75)	$4e^{-38}$
33	21518	21868	351	116	putative structural protein	putative structural protein phage LMA2 (116/116)	$1e^{-60}$
34	21870	22301	432	143	HP	HP of phage SN (141/143)	$1e^{-71}$
35	22311	22814	504	167	putative structural protein	structural protein phage SN (167/167)	$7e^{-90}$
36	22949	23353	405	134	putative structural protein	putative structural protein phage LMA2 (134/134)	$2e^{-73}$
37	23222	23353	132	43	putative structural protein	putative structural protein phage LMA2 (43/43)	$2e^{-16}$
38	23362	23955	594	197	putative structural protein	structural protein phage SN (180/183)	$1e^{-87}$

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
39	23965	24393	429	142	HP	HP of phage LBL3 (142/142)	$1e^{-78}$
40	24397	26973	2577	858	putative lytic tail protein (lysozyme like)	putative lytic tail protein phage LMA2 (858/858)	0.0
41	26973	27836	864	287	putative structural protein	putative structural protein phage LMA2 (284/287)	$1e^{-165}$
42	27836	28369	534	177	HP	HP of phage 14-1 (176/177)	$7e^{-99}$
43	28425	29090	666	221	putative baseplate protein	putative baseplate protein phage SN (217/221)	$7e^{-124}$
44	29101	30399	1299	432	putative baseplate protein (baseplate J)	putative baseplate protein phage SN (432/433)	0.0
45	30396	31910	1515	504	putative structural protein	putative structural protein phage LBL3 (500/504)	0.0
46	31915	34803	2889	964	putative tail fiber protein	putative tail fiber protein phage SN (904/962)	0.0
47	34805	35233	429	142	putative tail fiber component	putative tail fiber component phage PB1 (133/142)	$4e^{-72}$
48	35233	35895	663	220	putative endolysin (lysozyme like)	putative endolysin phage LMA2 (217/220)	$6e^{-126}$
49	35920	36171	252	83	HP	HP of phage 14-1 (83/83)	$1e^{-38}$
50	36451	37362	912	303	putative DNA ligase (DNA ligase A M)	putative DNA ligase phage SN (299/303)	$7e^{-177}$
51	37417	37971	555	184	HP (DUF 2166)	HP of phage 14-1 (179/184)	$4e^{-100}$
52	37968	38573	606	201	HP	HP of phage F8 (201/201)	$1e^{-108}$
53	38627	39523	897	298	HP	HP of phage 14-1 (297/299)	$3e^{-163}$
54	39612	40232	621	206	HP	HP of phage LMA2 (206/206)	$1e^{-113}$
55	40327	41886	1560	519	putative helicase (P-loop NTPase)	putative helicase phage LBL3 (509/519)	0.0

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
56	41883	42293	411	136	putative DNA helicase	putative DNA helicase phage SN (136/136)	$1e^{-73}$
57	42286	45393	3108	1035	putative DNA polymerase III alpha subunit (DNA pol3 alpha)	putative DNA polymerase III alpha subunit phage SN (1030/1035)	0.0
58	45393	45947	555	183	putative DNA polymerase III epsilon subunit (DnaQ like exo)	putative DNA polymerase III epsilon subunit phage LMA2 (181/184)	$4e^{-104}$
59	46023	47039	1017	388	HP	HP of phage 14-1 (337/338)	0.0
60	47042	47380	339	112	HP	HP of phage LMA2 (63/63)	$6e^{-28}$
61	47235	48152	918	305	putative thymidylate synthase (Thy1)	putative thymidylate synthase phage LBL3 (301/305)	0.0
62	48152	48358	207	68	HP	HP of phage 14-1 (68/68)	$2e^{-31}$
63	48366	48596	231	76	HP	HP of phage SN (76/76)	$2e^{-37}$
64	48629	48790	162	53	HP	HP of phage SN (53/53)	$3e^{-24}$
65	48831	49049	219	72	HP	HP of phage LBL3 (71/72)	$4e^{-32}$
66	49049	49279	231	76	HP	HP of phage 14-1 (76/76)	$4e^{-38}$
67	49367	50368	1002	303	HP	HP of phage 14-1 (333/333)	0.0
68	50473	51357	885	294	putative structural protein	putative structural protein phage LMA2 (277/293)	$2e^{-88}$
69	51519	52706	1188	395	putative ATP-dependent exonuclease V (P-loop NTPase)	putative ATP-dependent exonuclease V phage SN (395/395)	0.0
70	52693	53115	423	140	HP	HP of phage 14-1 (138/140)	$2e^{-75}$
71	53284	54069	786	261	HP	HP of phage 14-1 (260/261)	$8e^{-150}$
72 a	53996	55084	1089	362	HP	HP of phage 14-1 (312/314)	0.0

ORF	From	To	Length (bp)	Length (aa)	Predicted function (Protein superfamily)	Identity (aa identity)	e-value
72 b	55201	55383	183	60	HP	HP of phage 14-1 (60/60)	$6e^{-29}$
73	55407	55856	450	149	HP	HP of phage 14-1 (147/149)	$6e^{-82}$
74	55853	56929	1077	358	HP	HP of phage 14-1 (357/358)	0.0
75	56935	57120	186	61	HP	HP of phage LMA2 (61/61)	$2e^{-25}$
76	57268	59007	1740	579	putative primase (COG5545)	putative primase phage LMA2 (573/579)	0.0
77	59205	59786	582	193	HP	HP of phage F8 (116/146)	$1e^{-57}$
78	59800	59931	132	43	HP	no	
79	59977	60546	570	189	HP	HP of phage 14-1 (189/189)	$1e^{-107}$
80	60714	61325	612	203	HP	HP of phage 14-1 (203/203)	$2e^{-112}$
81	61515	62210	696	231	HP	HP of phage 14-1 (231/231)	$3e^{-130}$
82	62221	62532	312	103	HP	HP of phage 14-1 (103/103)	$5e^{-56}$
83	62585	62806	222	74	HP	HP of phage 14-1 (73/73)	$1e^{-33}$
84	62791	63072	282	93	HP	HP of phage 14-1 (63/63)	$6e^{-28}$
85	63123	63347	225	74	HP	HP of phage LBL3 (74/74)	$5e^{-36}$
86	63413	63739	327	108	HP	HP of phage LBL3 (108/108)	$8e^{-57}$
87	63740	64582	843	280	HP	HP of phage 14-1 (211/214)	$2e^{-120}$
88	64416	64625	210	69	HP	HP to phage 14-1 (69/69)	$4e^{-34}$
89	64622	64837	216	71	HP	HP of phage 14-1 (69/71)	$2e^{-32}$
90	64834	65058	225	74	HP	HP of phage LBL3 (64/66)	$3e^{-30}$
91	65031	65282	252	83	HP	HP to phage LBL3 (80/83)	$1e^{-40}$
92	65368	65541	174	57	HP	HP of phage LMA2 (41/57)	$4e^{-19}$
93	65559	66245	687	228	HP	HP of phage SN (147/226)	$2e^{-78}$

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